REMARKS

The notice of the change of the examiner is acknowledged.

Claims 1-5 and 39-58 are currently pending. Applicant draws the examiner's attention to the erroneously identified claims in the communication, on page 2, last paragraph: "Claims 1-6 remain rejected...", which should read "Claims 1-5 remain rejected...". The same error is found on page 8 of the communication.

Claim 1 is currently amended to incorporate the limitations of claim 4, and claim 4 is hereby canceled without prejudice or disclaimer.

Claim 5 is currently amended to make it depend from claim 1 and to substitute the term "Akt nucleic acid", as recited in claim 1, for the term "Akt molecule".

No new matter has been introduced.

Elected Subject Matter

The examiner has withdrawn claims 51-58 from consideration, which claims are drawn *inter alia* to a method of treating myocardial infarction comprising the step of administering to a subject in need of such treatment an Akt *polypeptide* in an amount effective to inhibit cardiac tissue necrosis in the subject, as allegedly being drawn to subject matter which has been previously withdrawn from consideration as being drawn to a non-elected invention. Applicant respectfully traverses.

Based on the restriction requirement mailed on August 30, 2000, Applicant elected with traverse Group I, corresponding to claims 1-5, drawn to a method of treating myocardial infarction by administering an Akt *molecule*, classified in class 514, subclass 2. As acknowledged by the examiner on page 3 of the Office Action, the specification teaches that "an Akt molecule" embraces, in pertinent part, both a *nucleic acid* encoding an Akt polypeptide and an Akt *polypeptide*. The definition of Akt molecule is provided, for example, on page 7 line 26 through page 8 line 12, as follows:

> An "Akt molecule", as used herein, embraces both "Akt nucleic acids" and "Akt polypepides" (discussed below). Akt molecules are capable of inhibiting apoptotic cell-death of a cell such as a cardiomyocyte, a skeletal myocyte or a vascular endothelial cell both *in vivo* and *in vitro*.
>
> An "Akt nucleic acid", as used herein, refers to a nucleic acid molecule which: (1) hybridizes under stringent conditions to a nucleic acid having the sequence of SEQ ID NO. 1 and (2) codes for an Akt polypeptide (i.e., a polypeptide that inhibits apoptotic cell-death of cells, and in particular, inhibits apoptotic cell-death of cardiomyocytes, skeletal myocytes and vascular endothelial cells. Preferably the Akt polypeptide maintains a serine-threonine kinase activity. The preferred Akt nucleic acid has the nucleic acid sequence of SEQ ID NO. 1. The Akt nucleic acids of the invention also include homologs and alleles of a nucleic acid having the sequence of SEQ ID NO. 1, as well as functionally equivalent fragments, variants, and analogs of the foregoing nucleic acids. "Functionally equivalent", in reference to an Akt nucleic acid fragment, variant, or analog, refers to a nucleic acid that codes for an Akt polypeptide that inhibits apoptotic cell-death of cells, and in particular, inhibits apoptotic cell-death of cardiomyocytes, skeletal myocytes and vascular endothelial cells. Preferably the Akt polypeptide maintains a serine-threonine kinase activity. More specifically, "functionally equivalent" refers to an Akt polypeptide that has a serine-threonine kinase activity and is capable of enhancing survival of a cell that may undergo apoptotic cell-death.

Thus, Applicant asserts that the recitation "an Akt molecule" as defined by the specification is meant to encompass both Akt nucleic acids and Akt polypeptides. Applicant reasonably relied on this definition in responding to the restriction requirement mailed on August 30, 2000. Accordingly, it is respectfully requested that the examiner reconsider the withdrawal of claims 51-58, extend consideration to the subject matter of these claims, and withdraw the finality of rejection.

Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 1-5 and 39-50 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement. According to the examiner, the specification, while enabling for "A method of treating a subject for myocardial infarction, comprising the step of: administering to the subject in need of such treatment a composition comprising a replication-defective adenovirus comprising a polynucleotide, wherein said composition is administered acutely

into the apical and anterolateral free wall of the heart, wherein said polynucleotide comprises a nucleotide sequence that encodes an Akt polypeptide, operatively linked to a promoter to promote expression of the Akt polypeptide in cardiomyocytes, wherein the Akt polypeptide comprises: the amino acid sequence of SEQ ID NO:2", does not reasonably provide enablement for the broadly claimed invention. The examiner states that the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to practice the invention commensurate in scope with these claims.

The examiner argues that the claims are broadly inclusive of treating myocardial infarctions via gene therapy or by simply administering any Akt polypeptide or nucleic acid in any manner and that "one can not extrapolate the teachings of the specification to the scope of the claims because applicant has not enabled *all* of these types of modified Akt proteins and nucleic acids for the treatment of myocardial infarctions" (emphasis added). Applicant respectfully asserts that the teachings of the specification, including but not limited to the working examples provided in the specification, are sufficiently enabling such that one of ordinary skill in the art can make and use the instant claimed invention. There is no requirement to provide a working example for any embodiments, let alone *all* of the embodiments. Nevertheless, the specification does disclose a number of embodiments of the claimed invention.

For example, Example 4 provides a working example of a method of treating a subject for myocardial infarctions using a replication-defective adenovirus, wherein a subject is administered a composition comprising an Akt polynucleotide which is administered acutely into the apical and anterolateral free wall of the heart. The examiner acknowledges that such embodiment is enabled as disclosed. Applicant disagrees that the invention is strictly limited to that particular exemplified embodiment, as suggested by the examiner. However, solely for the purpose of expediting the examination process, Applicant has, with traverse, amended the claims to incorporate some of the limitations suggested by the examiner.

Provided that the instant embodiment is enabled, it naturally follows that other embodiments, in which an Akt polynucleotide sequence encodes an Akt polypeptide that shares at least 98% sequence identity with that of SEQ ID NO:2, are also enabled. The specification provides sequences of Akt polynucleotides encoding Akt polypeptides that share at least 98% amino acid

identity with SEQ ID NO:2. The claimed invention teaches a method for treating myocardial infarction administering such Akt molecules *in an amount effective to inhibit cardiac tissue necrosis* in the subject. Therefore, it is understood by those skilled in the art that the biological activity of the Akt polypeptides sharing at least 98% amino acid identity with SEQ ID NO:2 as claimed is retained such that they are functionally equivalent to the Akt polypeptide of SEQ ID NO:2. Moreover, by the instant amendment it is specified that the Akt nucleic acid is administered acutely into cardiac tissue. Thus, each component of the method of treatment, e.g., the composition, the target subject, the amount, the location of administration, and the route/mode of administration, is provided. Accordingly, Applicant believes that the enablement requirement is met.

In addition, the enabling delivery method should not be limited to a replication-defective adenovirus, which was taught merely as an example, as suggested by the examiner. Applicant respectfully draws the examiner's attention to the specification, for instance, on page 5 lines 12-15, as well as throughout pages 11-13, wherein a number of examples of delivery methods are described. The specification provides preferred expression vectors for mediating nucleic acid delivery, such as adenoviral vector, modified adenoviral vector, retroviral vector, plasmid, liposome. Applicant asserts that at the time of filing of the instant application, these various delivery methods were known in the art and that experimental protocols for making them and using them were readily available.

For example, San et al. used cationic lipids as a non-viral delivery vector to form DNA-liposome complexes for use *in vivo* (San H et al., 1993, *Hum Gene Ther* 4:781-8). Similarly, Zhu et al. successfully demonstrated systematic gene expression after intravenous DNA delivery into adult mice by injection of expression plasmid:liposome complexes (Zhu N et al., 1993, *Science* 261:209-11). These examples provide support for the recitation of cationic lipids on page 13, last paragraph:

> Liposomes are commercially available from Gibco BRL, for example, as LIPOFECTIN™ and LIPOFECTACE™, which are formed of cationic lipids such as N-[1-(2, 3 dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB).

Gal et al. showed direct myocardial transfection in two animal models, in which pure untreated DNA was injected into skeletal and cardiac muscles (Gal D et al., 1993, *Lab Invest* 68:18-25). The specification also provides, on page 10, last paragraph:

> The Akt nucleic acids of the invention can be delivered to the preferred cell types of the invention *alone* or in association with a vector" (Emphasis added).

Thus, the recitation is supported by general knowledge in the art at the time of the filing that nucleic acid delivery is feasible by various means.

Similarly, Coffin et al. reported in 1996 that herpes simplex virus 1 (HSV1) can be successfully employed for gene delivery in the rat heart *in vivo* (Coffin RS et al., 1996, *Gene Ther* 3:560-6). Moreover, in the study published by Prentice et al. in 1996, it was demonstrated that ischemic/reperfused myocardium can express recombinant protein following direct DNA or retroviral injection (Prentice H et al., 1996, *J Mol Cell Cardiol* 28:133-40). The specification provides examples of viruses that can be used to deliver Akt molecules; see for example page 11, starting at line 16, which reads:

> Viral vectors are a preferred type of vector and include, but are not limited to, nucleic acid sequences from the following viruses: adenovirus; adeno-associated virus; retrovirus, such as moloney murine leukemia virus; harvey murine sarcoma virus; murine mammary tumor virus; rouse sarcoma virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus.

Thus, a number of published studies established, prior to the filing of the instant application, that in various animal models gene delivery, as well as expression of foreign proteins, was feasible and effective by the use of any of a variety of delivery methods, including replication-defective adenovirus, modified adenovirus, retrovirus, herpes simplex virus, liposomes, and purified plasmid. Therefore, while a working example is provided that used adenovirus as a vector for Akt delivery as one embodiment, the claimed invention is not limited to that particular embodiment.

Furthermore, according to Feldman and Steg (*Cardiovascular Res* 35:391-44; 1997), cardiovascular gene therapy is no longer a prophetic therapy but "a clinical reality". The following passage appears in their review article published in 1997:

> Cardiovascular gene therapy is becoming a clinical reality due to improved vectors, delivery systems and careful experimental validation studies. Nearly all cardiovascular diseases are amenable to gene therapy, but the optimal combination of vector, delivery system and therapeutic gene is likely to be unique to each application. Currently, the most efficient vectors available are replication-defective adenoviral vectors, but transgene expression is limited in time due to a strong immune response. Conversely, non-viral vectors or plasmid DNA may be used safely but have very limited efficiency. Percutaneous, catheter-based delivery is feasible for most applications. The ultimate issues that will decide of the future of gene therapy are safety of the transfer and delivery techniques as well as cost/effectiveness comparisons with alternative therapies, including local delivery of drugs, proteins and/or mechanical devices. (Abstract)

Thus, these methods were publicly known and readily available to those skilled in the art at the time of filing of the instant application. Optimization is not the proper standard for enablement. Optimization *per se* should not in any case be considered "undue experimentation", for a person skilled in the art would appreciate the need for optimizing to each application and that it is within routine practice inherent to the art. It is understood that optimal conditions for use are to be determined so as to tailor to factors involved in each case, including but not limited to co-existing conditions, circumstances of treatment, age of the subject, other health issues and cost. In other words, these issues relate to clinical and business aspects but not technical concerns.

With regard to the reference by Eck et al., cited by the examiner, Applicant asserts that the discussion in that reference pertains to a standard relevant to clinical applications, a standard that extends well beyond what is necessary to satisfy the enablement requirement under 35 U.S.C. § 112, first paragraph. Indeed, the discussion in Eck et al. pertains to the domain of the Food and Drug Administration (FDA). In fact, the authors do not deny the realistic possibility of gene therapy, but cautiously outline technical hurdles that may exist before such therapies can be considered safe and be available for wider use for treating human subjects at the level of the FDA safety standard. Furthermore, Applicant points out that the Eck reference was taken from a chapter of a textbook

published in 1996. It is commonly recognized that information contained in a textbook is often outdated by 2-3 years by the time of its publication. Therefore, it is not reasonable to assume the teachings of Eck et al. accurately represent the state of the art in 1996, when the textbook was published.

In arguing that numerous factors complicate gene therapy, the Examiner also cited Rubanyi (2001). Applicant respectfully asserts that the standard held forth by Rubanyi is "convincing clinical efficacy" of human gene therapy (see Abstract). Again, this is a standard that extends well beyond what is required to meet the enablement requirement under 35 U.S.C. § 112, first paragraph. Nevertheless, even at this high level of standard, the author states that "the most promising areas for gene therapy today are...cardiovascular diseases (more specifically, ... myocardial ischemia...)" (Abstract, 4th paragraph). The author further states that "existing gene delivery technologies may be sufficient to achieve effective and safe therapeutic benefits for some of these indications" (Abstract). Therefore, the cited reference actually supports Applicant's position that the enablement requirement is met.

Taken together, Applicant believes that the claimed invention is enabled as disclosed. Reconsideration is thus respectfully requested.

In view of the foregoing, Applicant respectfully requests that the examiner reconsider and withdraw the rejection made under U.S.C. § 112, first paragraph, for alleged lack of enablement.

Applicant believes the pending application is in condition for allowance, apart from current pendency of withdrawn claims. Favorable response is respectfully requested.

Respectfully submitted,

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Gene delivery to the heart in vivo and to cardiac myocytes and vascular smooth muscle cells in vitro using herpes virus vectors

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Herpes simplex virus 1 (HSV1), while usually thought of as neurotrophic, can also efficiently infect a wide variety of non-neuronal cell types and so might be developed as a vector for gene delivery to non-neuronal as well as neuronal cells. Here we have tested three different disabled HSV vectors for their ability to deliver a lacZ gene to primary cardiac myocytes and vascular smooth muscle cells in vitro, and used the most efficient virus to transfect the rat heart in vivo. We also assessed the degree of cytopathic effect of the various viruses on the cardiac myocytes in vitro by testing the effects on the frequency of beating in synchronously beating myocyte cultures. While an HSV mutant in which the essential immediate–early gene IE2 had been deleted gave high efficiency gene transfer to the cardiac myocytes in vitro and the rat heart in vivo, viruses in which ICP34.5 or ICP34.5 and VMW65 were inactive (and which were also unable to replicate in these cells) gave a much lower efficiency of gene transfer, mirroring the degree of cytopathic effect observed in the beating myocyte cultures. Gene transfer to the vascular smooth muscle cells was considerably less efficient than to the myocytes in all cases. These results indicate that while HSV may be inappropriate for highly efficient gene transfer to the arterial wall, efficient gene transfer can be achieved in the myocardium, and thus that HSV vectors may be suitable for the alteration of cardiac cell physiology in vivo.

Keywords: *herpes virus vectors; cardiac myocytes; vascular smooth muscle cells; gene therapy*

Introduction

A variety of methods are under development for gene transfer to the myocardium and coronary vasculature, including the use of adenoviral vectors and the direct injection of plasmid DNA (reviewed by Nabel[1]), where it is hoped that they may in the future allow the treatment of a number of cardiovascular diseases through the expression of therapeutic gene products. Example conditions for which gene therapeutic protocols can be envisaged include ischaemic heart disease or in restenosis prevention following percutaneous transluminal coronary angioplasty.

Herpes simplex virus 1 (HSV1) has been proposed as a candidate vector for gene delivery to the nervous systems (reviewed by Coffin and Latchman[2]) as it can produce a lifelong latent infection in neurons. However, HSV1 can also infect a wide range of other cell types and could therefore be used for gene delivery to non-neuronal cells as long as lytic infection were inhibited. Indeed HSV has recently been used to deliver *lacZ* to mouse smooth muscle cells *in vitro* and *in vivo*.[3] Moreover the particularly stable DNA structure formed by nonreplicating HSV genomes may be particularly suitable for allowing long-term genetic correction in nondividing cells. HSV is highly pathogenic and thus must, like most other viral vectors, be disabled in some way. Most replication defective HSV-1 vectors contain a deletion to remove one or more immediate–early (IE) genes in order to prevent virus replication at the earliest possible time after infection. These genes must be complemented *in trans* for growth in culture. Alternatively, either an inactivating mutation in the gene encoding VMW65 (which transactivates IE genes after infection) can be used to produce a nonpathogenic virus, or genes necessary for replication in particular target cell types can be removed. A mutation of this last type is provided by removal of the ICP34.5 gene which prevents replication in some fully differentiated cell types (including neurons) but which still allows virus growth in actively dividing fibroblasts in culture.[4] Here we have tested disabled viruses of these (or combinations of the three) types for the ability to direct β-galactosidase (*lacZ*) expression in cultured rat cardiac myocytes, primary rat aortic vascular smooth muscle cells (VSMCs), and a VSMC cell-line *in vitro*, and also tested the most efficient virus in the rat heart *in vivo*. These viruses were either deleted for ICP34.5, ICP34.5 with VMW65 inactivated, or deleted for IE2 (encoding essential immediate–early protein ICP27). Thus while

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wild-type HSV is highly neurotrophic, these various deletions of the HSV genome would prevent any possible neurological complications when using such vectors for gene delivery to non-neuronal cells. However, while ICP34.5-deleted viruses have already been tested and been shown to give safe transgene delivery to the mouse and rat central and peripheral nervous systems *in vivo* (manuscript submitted), their efficiency for gene delivery, their replication status and the degree of cytopathic effect (CPE) produced in cardiac cells or VSMCs were unknown at the begining of this study.

Results

Experiments were performed both to assess the efficiency of gene transfer to the cardiomyocytes and vascular cells and to assess the degree of cytotoxicity of the various viruses. Thus cell cultures were infected with a number of viruses carrying the *lacZ* gene: a nondisabled virus (BE8),[5] viruses disabled by the removal of ICP34.5[6] or ICP34.5 together with the inactivation of VMW65[7] (1716/*lacZ* or 1764/*lacZ*), or a virus with the essential IE gene encoding ICP27 inactivated (27-*lacZ*).[8] Cells were then either stained with X-gal at 1 or 3 days and blue cells counted as a means of assessing the efficiency of gene delivery, or the health of the culture roughly assessed by counting the frequency of beating (beats per min) in synchronously contracting myocyte cultures, as compared with an uninfected control at 1 day, 2 days and 5 days after infection. Healthy, recently isolated cultures of rat newborn cardiomyocytes contract in a regular and synchronous fashion for a number of days, although they are highly sensitive to physiological or other insult during this time. The degree of virus replication was assessed by removal of the supernatant from the cultures and titration (followed by plaque counting) on a susceptible cell line (BHK C-13, or BHK C-13 expressing ICP27).

X-gal staining

Bearing in mind the beat frequency data below (see Table 1), which shows that at multiplicities of infection (MOI) >1 there is considerable cytotoxicity even for the disabled viruses, the X-gal staining experiments were all performed at MOI = 1. As can be seen from Figure 1a, all the viruses can deliver *lacZ* to the cardiomyocytes, although in accordance with the beat frequency data below, the efficiency of gene transfer varies significantly. However as in each case (except for BE8) there was little difference between the numbers of blue cells after 1 day or 3 days, it appeared likely that all the disabled viruses were incapable of replication in the cardiomyocytes. This was confirmed by the virus titration experiments which showed that while large numbers of plaques were generated on BHK cells by the supernatant harvested from cardiomyocytes 3 days after inoculation with BE8, no plaques were generated from the supernatant after inoculation with 1716/*lacZ*, 1764/*lacZ* or 27-*lacZ* (in this last case titrated on a BHK cell line expressing ICP27).

The nondisabled virus (BE8) gave a high proportion of stained cells, but with a high degree of accompanying cell death, showing not only the ability of HSV to infect and replicate in cardiomyocytes, but also the requirement for effective disablement of an HSV vector. However, while the viruses with either ICP34.5 or ICP34.5 and VMW65 removed gave only a relatively low number of blue staining cells, 27-*lacZ* produced both a very high percentage of blue cells and little visible CPE. This suggests that the absolute block to replication provided by this mutant, and the probable lower level of virus gene expression is more effective at generating an efficient and non-cytopathic vector for cardiac cells than removal of ICP34.5 or ICP34.5 and VMW65. This difference may be due to the specific function of ICP34.5 in some cell types (see Beat frequencies and Discussion) which might prevent efficient *lacZ* expression.

While the primary rat cardiomyocytes were evidently highly infectable by HSV (either wild-type or disabled), the primary aortic vascular smooth muscle cells were considerably less susceptible, as even the nondisabled virus gave only a considerably lower percentage of X-gal staining cells even at higher multiplicities of infection than used for the cardiocytes above (MOI of 5 instead of 1; Figure 1b). However, of the disabled viruses 27-*lacZ* again gave the best results, with only a few cells staining with X-gal when infected with the ICP34.5 or ICP34.5/VMW65 deleted virus (again at MOI = 5). While these primary cultures of vascular smooth muscle cells could be infected by HSV, albeit at lower efficiency than the cardiomyocytes, the vascular smooth muscle cell line used gave no blue cells even at MOI > 10 with 27-*lacZ*, and only a few blue cells when infected with BE8 at high MOI. HSV is therefore inappropriate for gene delivery to these cells in culture.

Beat frequencies

Table 1 shows the results of the beat frequency experiments after inoculation of the cardiomyocytes with the various viruses at multiplicities of infection (MOI) of 0.5, 1 and 5. The nondisabled virus (BE8) showed marked cytotoxicity after 1 day, with little and irregular beating at MOI = 1 and no beating at MOI = 5. By day 2, some recovery had occurred at MOI = 1 although beating was slightly higher than mock-infected frequencies (approximately 200, as against approximately 150) and still irregular. All the cells were dead by day 5. 1716/*lacZ* and 1764/*lacZ* showed beat frequencies at near to mock-infected levels at MOI $\leq$ 1 after 1 day, although at higher multiplicities of infection beat frequency was reduced. At day 2, the cells infected at lower multiplicity were still beating although at below mock levels, while with the higher multiplicities beating had essentially stopped. The cells were again dead by day 5. The removal of ICP27 gave a considerable improvement in cell survival and beat frequency at MOI $\leq$ 1, with at day 1 near to mock levels, day 2 half way between mock and 1716/*lacZ* or 1764/*lacZ* levels, and at day 5 the cells were still beating slowly. At higher MOI, beat frequencies were more markedly reduced at all time-points. However, at day 5 even mock-infected cultures are no longer beating stably, with high speed, unsynchronised contractions (fibrillation).

These results show that while all the disabled viruses are incapable of replication in the cardiomyocytes (see above), only the removal of ICP27 generates a vector in which the toxic effects of infection have been reduced significantly, as at an MOI $\leq$ 1 beat frequencies are similar to mock-infected controls. The deletion of ICP34.5 or ICP34.5 and VMW65, while preventing replication, does not provide a vector in which the cytotoxic effects of infection have been very significantly reduced (although the effects are considerably lower than with BE8 which

(a)

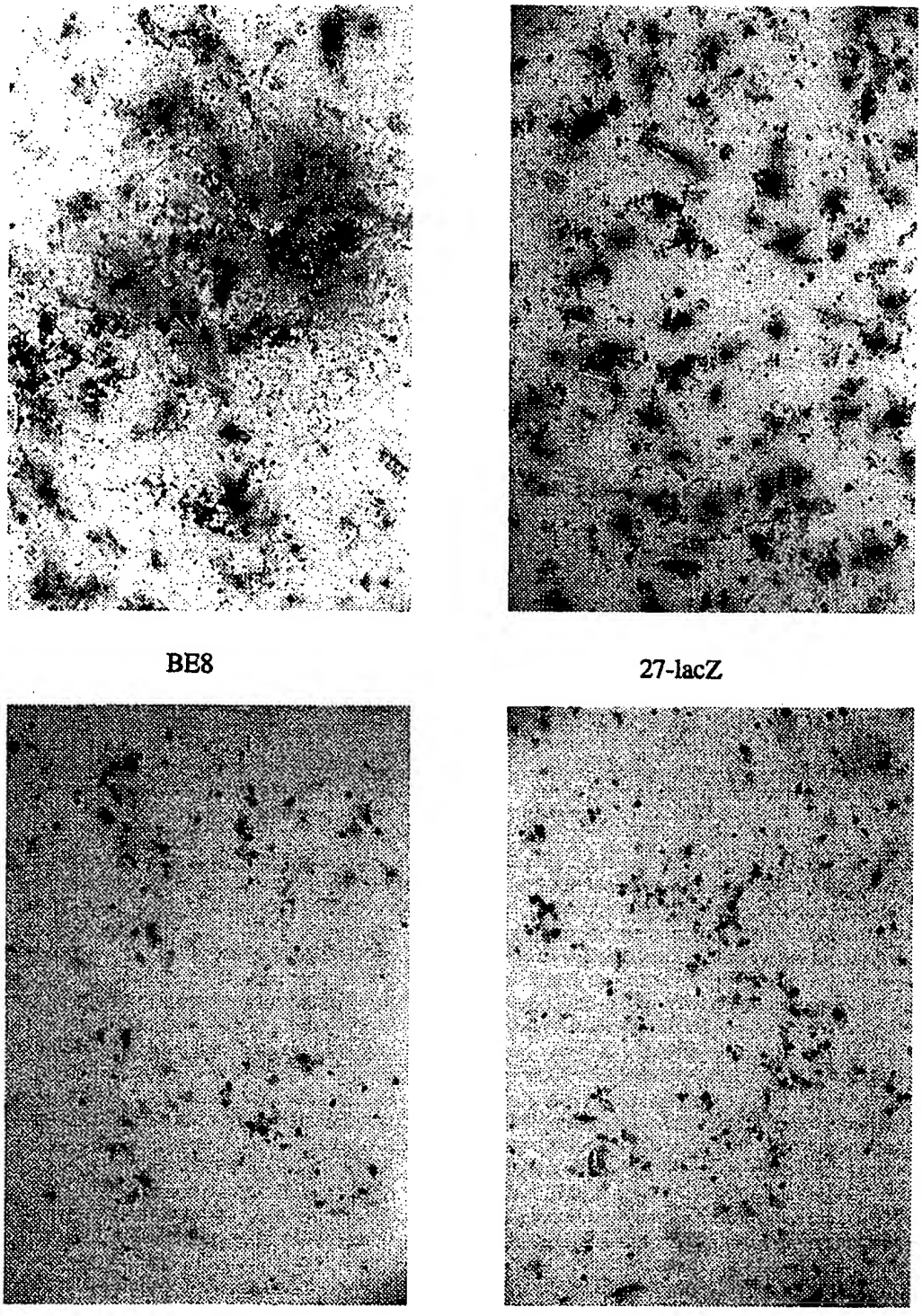
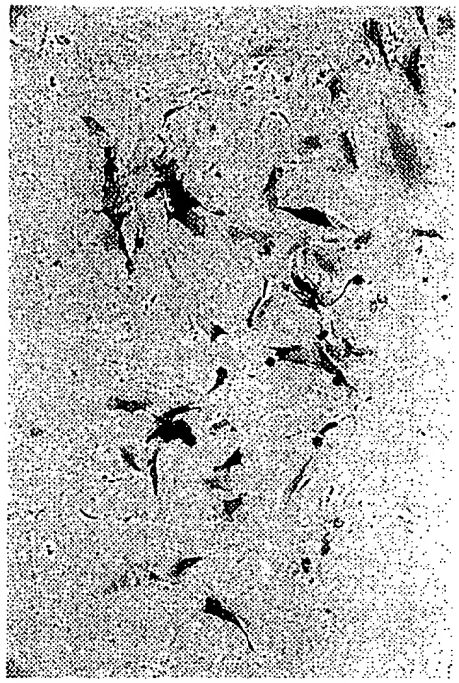
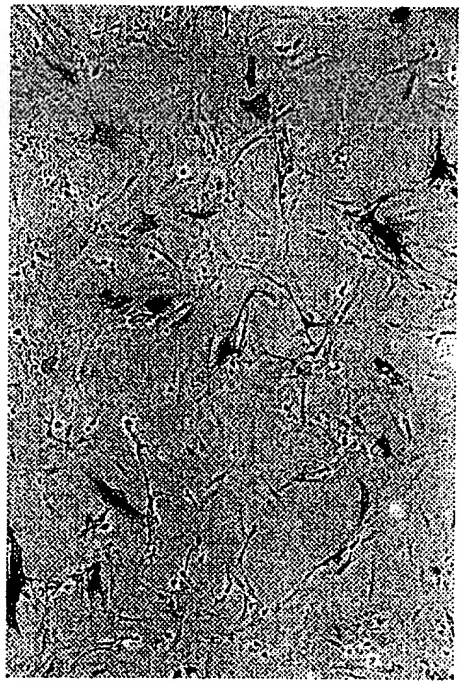


Figure 1 *(a) Newborn rat primary cardiac myocyte cultures or (b) primary vascular smooth muscle cultures stained with X-gal 3 days after infection with either BE8, 27-lacZ, 1716/lacZ or 1764/lacZ. The primary cardiac myocytes were infected at MOI = 1 and the vascular smooth muscle cells at MOI = 5.*

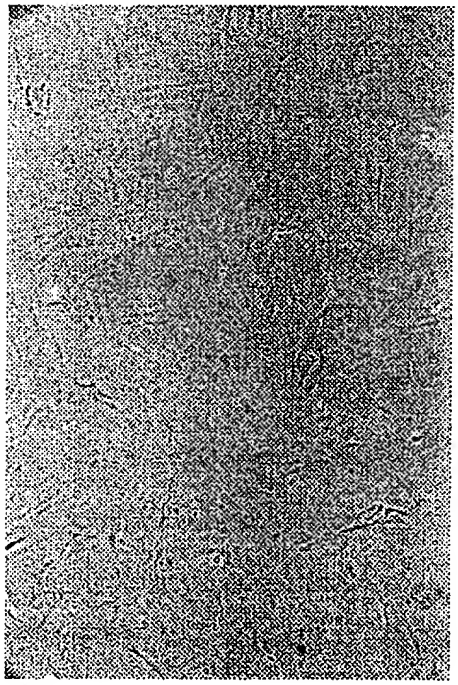
(b)



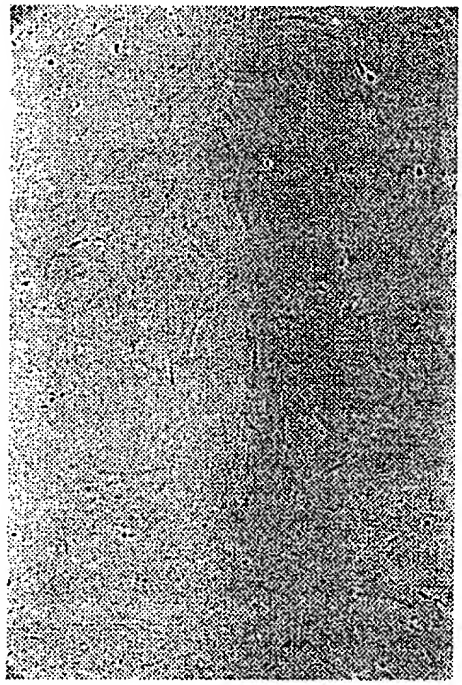
BE8



27-lacZ



1716/lacZ



1764/lacZ

Figure 1 (*Continued*).

Table 1 Frequency of synchronous beating in newborn rat primary cardiac myocyte cultures 1 day, 2 days or 5 days after infection with either BE8, 1716/*lacZ*, 1764/*lacZ* or 27-*lacZ* at various MOIs

MOI	*BE8*		*1716/lacZ*			*1764/lacZ*			*27-lacZ*			*Mock*
	1	*5*	*0.5*	*1*	*5*	*0.5*	*1*	*5*	*0.5*	*1*	*5*	
Day 1	8 irregular	0	162	145	43	164	158	62	160	163	100	160
Day 2	204 irregular	0	48	40	7 irregular	52	47	5 irregular	150	135	58	153
Day 5	0	0	0	0	0	0	0	0	100	50	0	276 fibrillating

gives a lytic infection in these cells). This might be for two reasons: firstly removal of ICP34.5 (and VMW65 in the case of 1764/*lacZ*) may still allow the expression of many potentially cytotoxic HSV gene products, particularly the IE genes, which may account for the effects we have observed, or secondly in the absence of ICP34.5 protein synthesis might be shutdown in the infected cells which might also account for the effects on beat frequency. This type of response has been noted previously in a neuronally derived cell line[9] (although it is not seen in other nonpermissive cells),[10,11] where it was suggested that the natural response to infection by an ICP34.5-deleted virus in these cells was a shutdown in protein synthesis, possibly leading to apoptosis, which would limit the extent of the infection in the host, but which was blocked by the expression of ICP34.5.

In vivo gene delivery to the heart

To assess whether the apparent utility of HSV vectors for the heart *in vitro* was reflected in efficient delivery to the heart *in vivo*, the most successful mutant *in vitro* (deleted for ICP27) was used *in vivo* in the rat heart. For these experiments intramyocardial injections were made directly through the chest wall of anaesthetised adult Lewis rats after locating the heart by palpation. The rats were allowed to recover and after 2 days were killed, the heart removed, fixed and stained with X-gal. As can be seen in Figure 2 efficient gene delivery can be achieved in the adult rat myocardium as large areas staining with X-gal can be seen around the inoculated site. The rats remained healthy during the 2-day incubation period and the X-gal stained areas remained healthy in appearance with no apparent alteration in cell morphology suggesting any cytopathic effects of the inoculated virus to be minimal. However, as would be expected from a nonreplicating viral vector, the area of staining is limited to the immediate vicinity of the inoculated site, suggesting that multiple injections or other means of delivery may be required for significant physiological effects if such a vector were to be used for the delivery of, for example, genes for the 70 kDa heat shock protein (HSP70) which might protect against the effects of ischaemia.[12,13]

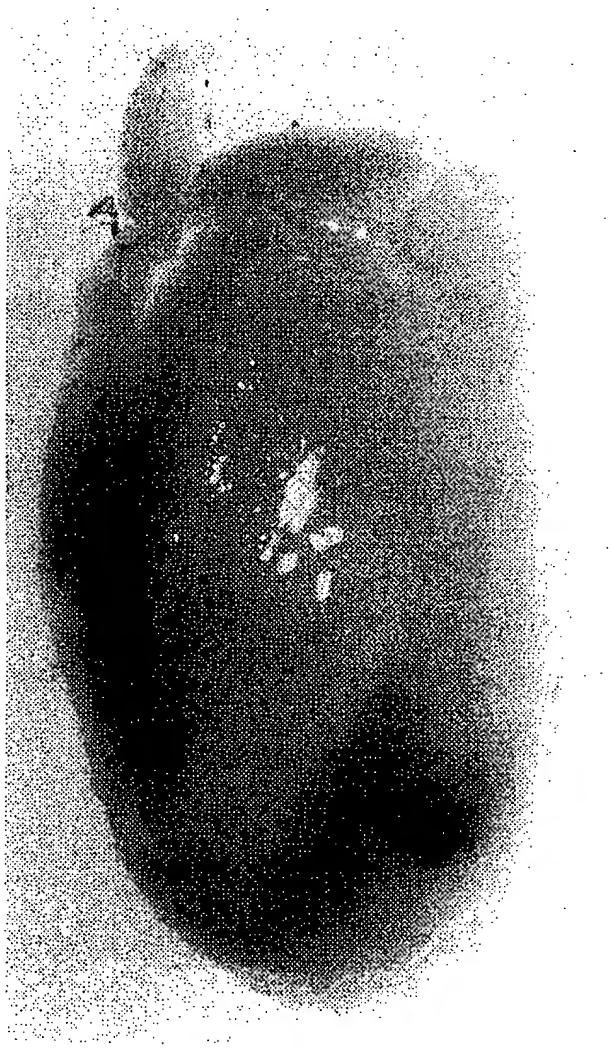


Figure 2 In vivo gene delivery to the rat myocardium. Rat hearts were inoculated as described in the text and fixed and stained with X-gal 2 days after inoculation with 27-lacZ.

Discussion

HSV has traditionally been regarded as an ideal candidate vector for the nervous system, as with suitable development its neurotrophic life-style and its ability to

maintain a lifelong latent infection might provide a means by which neuronal physiology could be altered in the long term after a once-only application of such a vector system. However HSV can also efficiently infect many other cell types and in some circumstances might provide specific advantages over other vector systems, particularly in the delivery of genes to terminally differentiated cells such as those of the myocardium, where very long-term gene expression from latent virus might be envisaged, or where large genetic insertions might be required. HSV does not have the packaging constraints of other viruses, potentially allowing the use of large control DNA regions which might be important for correct cell type-specific gene expression. Indeed HSV has recently been shown to be efficient at delivering *lacZ* to skeletal muscle cells *in vivo*.[3] However, to exploit HSV as a vector it must be disabled to minimise the effects on the target cell, and a number of strategies for this have previously been employed. These have included the deletion of essential genes, allowing virus growth only in a complementing cell line and the deletion of genes which specifically prevent growth in target cells but still allow growth in culture. For neurons these have included genes such as ICP6,[14] thymidine kinase[15] and ICP34.5,[4] only the last of which provides an absolute block to a productive infection in these cells. Here we have tested and compared viruses disabled by both these means and showed that while viruses deleted for the essential IE gene ICP27 (and therefore grown on ICP27 expressing cells) provide efficient and relatively noncytopathic vectors for cardiomyocytes *in vitro* and the rat myocardium *in vivo*, deletion of ICP34.5, while preventing replication in these cells, does not allow efficient gene delivery. We have also shown that *in vitro* HSV is less efficient for gene transfer to VSMCs, although this was not tested *in vivo*.

It has been speculated that delivery of a number of genes to the myocardium, particularly HSPs, might protect against the effects of ischaemia,[12] and indeed transgenic mice overexpressing HSP70 are significantly protected.[13] It has also been shown that fibroblast growth factor (FGF) when delivered to porcine iliofemoral arteries can induce angiogenesis.[16] While a number of vector systems have been tested for *in vitro* or *in vivo* gene delivery, including adenoviral vectors, retroviral vectors, liposomes or the direct injection of plasmid DNA (reviewed by Nabel[1]), none provides an optimal gene delivery system under all circumstances. HSV has previously been shown to allow delivery of *lacZ* in a nonvascularised cardiac transplantation model in the mouse where neonatal whole hearts were removed, inoculated with virus, and placed subcutaneously in the ear pinna of recipient mice.[17] Our experiments further show that HSV could be developed as a vector system to deliver some or all of these genes to the myocardium, and that it might provide advantages in some circumstances, for example for long-term or tissue-specific expression. For these reasons we are currently developing herpes vectors to express a number of HSPs and FGF to allow the potential of HSV for altering cardiac cell physiology to be tested *in vivo*.

Materials and methods

Cardiocyte cell culture

Ventricular myocytes from the hearts of <2-day-old neonatal Sprague–Dawley rats were isolated and cultured as previously described.[18–20] Cardiocyte cultures under these conditions start to beat in synchrony within 24–48 h of isolation, the percentage of beating cells exceeding 85% for the duration of the experiment. Cells grown on laminin-coated coverslips were used for immunofluorescent staining with an antimyosin antibody (Amersham International, Amersham, UK) to confirm the proportion of cardiocytes staining for myosin heavy chain.

Vascular smooth muscle cell (VSMC) cultures

Primary VSMCs were produced by removal of the thoracoabdominal aortas of 3-month-old male Wistar rats which were then stripped of endothelium and adventitia. Medial VSMCs were obtained by the combined collagenase/elastase digestion method.[21] Isolated cells were maintained in Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum, 50 mg/ml penicillin, 50 mg/ml streptomycin (all Gibco, Gaithersburg, MD, USA) at 37°C with 5% CO_2. VSMCs were identified by their characteristic morphology and immunostaining for sm-myosin (M-7648 from Sigma, Poole, UK). At confluence, cells were passaged using 0.25% trypsin, 3 mM EDTA and were used at passage 4–6 for these experiments. The VSMC cell line used is an SV40-transformed rat abdominal aorta smooth muscle culture obtained from the American Type Culture Collection (ATCC-CRL2018, Rockville, MD, USA).

Virus stocks

Herpes virus strains used were BE8 (an essentially wild-type virus with a CMV IE promoter/*lacZ* insertion into a nonessential gene (US5))[5] and three disabled viruses 1716/*lacZ*, 1764/*lacZ* and 27-*lacZ*.[8] 1716/*lacZ* and 1764/*lacZ* were produced by the insertion of a chimaeric herpes latently active transcript (LAT)-Moloney murine leukaemia virus long terminal repeat (MoMLVLTR) promoter/*lacZ* cassette into the UL43 gene of HSV1 strains 1716 and 1764 respectively, by standard methods.[2] 1716 Is deleted for both copies of ICP34.5,[6] and 1764 also has an inactivating insertional mutation in the gene for VMW65.[7] UL43 is a nonessential HSV gene, unnecessary for growth in culture and which does not affect the kinetics of the establishment or reactivation from latency *in vivo*.[22] 27-*lacZ* has an ICP6 promoter/*lacZ* insertion into the gene encoding the essentail IE protein ICP27.[8]

In vivo inoculation

Approximately 20 μl intramyocardial injections of 27-*lacZ* (5×10^8 p.f.u./ml) were made directly through the chest wall of anaesthetised adult Lewis rats after accurately locating the heart by palpation. The rats were allowed to recover and after 2 days were killed, the heart removed, fixed and stained with X-gal.

X-gal staining

Culture medium was removed from the cells which were then fixed in 4% paraformaldehyde at room temperature for 15 min. After washing twice in phosphate buffered saline (PBS), cells were then stained with X-gal for approximately 1 h at 37°C in 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM magnesium chloride, 0.02% NP40, 0.02% sodium deoxycholate, 1 mg/ml X-gal (Sigma, Poole, UK) in PBS. Whole hearts inoculated *in vivo* were removed from the animal after death

and treated similarly except that the fixing step was extended to 1 h and incubation in X-gal to 4 h.

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References

1 Nabel EB. Gene therapy for cardiovascular diseases. *Circulation* 1995; 91: 541–548.
2 Coffin RS, Latchman DS. Herpes simplex virus-based vectors. In: Latchman DS (ed). *Genetic Manipulation of the Nervous System*. Academic Press: London, 1996, pp 99–111.
3 Huard J, Goins WF, Glorioso JC. Herpes simplex virus type 1 vector mediated gene transfer to muscle. *Gene Therapy* 1995; 2: 385–392.
4 Chou J, Kern ER, Whitley RJ, Roizman B. Mapping of herpes simplex virus-1 neurovirulence to γ34.5, a gene nonessential for growth in culture. *Science* 1990; 250: 1262–1266.
5 Balan P *et al*. An analysis of the *in vitro* and *in vivo* phenotypes of mutants of herpes simplex virus type 1 lacking glycoproteins gG, gE, gI or the putative gJ. *J Gen Virol* 1994; 75: 1245–1258.
6 MacLean AR *et al*. Herpes simplex virus type 1 deletion variants 1714 and 1716 pinpoint neurovirulence related sequences in Glasgow strain 17+ between immediate–early gene 1 and the 'a' sequence. *J Gen Virol* 1991; 72: 632–639.
7 Ace CI *et al*. Construction and characterisation of a herpes simplex virus type 1 mutant unable to transinduce immediate–early gene expression. *J Virol* 1989; 63: 2260–2269.
8 Smith IL, Hardwicke MA, Sandri-Goldin RM. Evidence that the herpes simplex virus immediate–early protein ICP27 acts post-transcriptionally during infection to regulate gene expression. *Virology* 1992; 186: 74–86.
9 Chou J, Roizman B. The γ34.5 gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. *Proc Natl Acad Sci USA* 1992; 89: 3266–3270.
10 Brown SM *et al*. Cell type and cell state determine differential *in vitro* growth of non-neurovirulent ICP34.5-negative herpes simplex virus types 1 and 2. *J Gen Virol* 1994; 75: 2367–2377.
11 Brown SM, MacLean AR, Aitken JD, Harland J. ICP34.5 influences herpes simplex virus type 1 maturation and egress from infected cells *in vitro*. *J Gen Virol* 1994; 75: 3679–3686.
12 Marber MS, Walker JM, Latchman DS, Yellon DM. Myocardial protection after whole body heat stress in the rabbit is dependent on metabolic substrate and is related to the amount of the inducible 70-kD heat stress protein. *J Clin Invest* 1994; 93: 1087–1094.
13 Marber MS *et al*. Overexpression of the rat inducible 70 kilodalton heat stress protein in a transgenic mouse increases the resistance of the heart to ischaemic injury. *J Clin Invest* 1995; 95: 1446–1456.
14 Goldstein D, Weller SK. Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: isolation and characterisation of an ICP6 *lacZ* insertion mutant. *J Virol* 1988; 62: 196–205.
15 Coen DM *et al*. Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate. *Proc Natl Acad Sci USA* 1989; 86: 4736–4740.
16 Nabel EG *et al*. Recombinant FGF-1 promotes intimal hyperplasia and angiogenesis in arteries *in vivo*. *Nature* 1993; 362: 844–846.
17 Qin L *et al*. Multiple vectors effectively achieve gene transfer in a murine cardiac transplantation model. *Transplantation* 1995; 59: 809–816.
18 Chien KR *et al*. Release of arachidonate from membrane phospholipids in cultured neonatal rat myocardial cells during adenosine triphosphate depletion. *J Clin Invest* 1985; 75: 1770–1780.
19 Buja LM *et al*. Alterations of ultrastructure and elemental composition in cultured neonatal rat cardiac myocytes after metabolic inhibition with iodoacetic acid. *Lab Invest* 1988; 53: 397–412.
20 Knowlton KU *et al*. Co-regulation of the atrial natriuretic factor and cardiac myosin light chain-2 genes during a adrenergic stimulation of neonatal rat ventricular cells. *J Biol Chem* 1991; 266: 7759–7768.
21 Rennick RE, Cambell JW, Cambell GR. Vascular smooth muscle phenotype and growth behaviour can be influenced by macrophages *in vitro*. *Atherosclerotion* 1988; 71: 35–43.
22 MacLean CAS *et al*. Investigation of herpes simplex virus type 1 genes encoding multiply inserted membrane proteins. *J Gen Virol* 1991; 72: 897–906.

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Review

Optimal techniques for arterial gene transfer

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Abstract

Cardiovascular gene therapy is becoming a clinical reality due to improved vectors, delivery systems and careful experimental validation studies. Nearly all cardiovascular diseases are amenable to gene therapy, but the optimal combination of vector, delivery system and therapeutic gene is likely to be unique to each application. Currently, the most efficient vectors available are replication-defective adenoviral vectors, but transgene expression is limited in time due to a strong immune response. Conversely, non-viral vectors or plasmid DNA may be used safely but have very limited efficiency. Percutaneous, catheter-based delivery is feasible for most applications. The ultimate issues that will decide of the future of gene therapy are safety of the transfer and delivery techniques as well as cost/effectiveness comparisons with alternative therapies, including local delivery of drugs, proteins and/or mechanical devices. © 1997 Elsevier Science B.V.

Keywords: Gene therapy; Atherosclerosis; Adenovirus; Liposomes; Catheter; Restenosis; Angiogenesis; Plaque stabilization

1. Introduction

Atherosclerosis and its complications represent the first cause of mortality and morbidity in the western world [1]. Major advances have been made in the treatment and prevention of symptomatic atherosclerosis. Improved understanding of the pathophysiology of atherosclerosis and its complications [2], as well as spectacular advances in the molecular biology of the vascular wall [3,4] may open new perspectives for treatment based upon local delivery of genetic material designed to modify the atherosclerotic plaque at the molecular level [5]. Transfer of a functional gene into arterial wall cells, termed arterial gene therapy, may be used to replace or palliate a defective gene, or to express a protein with a therapeutic effect [6].

Effective arterial gene therapy requires techniques to introduce (transfect) a foreign gene (transgene) into the cells of the arterial wall. These techniques rely on transfer vectors which facilitate cellular penetration and intra-cellular trafficking of the transgene, as well as local delivery systems, either catheter-based or surgical, to deliver the vector to the vicinity of the target cells.

2. Vectors

Under certain conditions, it is feasible to introduce foreign DNA into the nucleus of eukaryotic cells. This has been used to obtain transient or stable expression of several genes in cell lines. To achieve expression of foreign DNA, however, the transferred gene should enter the cell, escape degradation by lysosomal enzymes, cross the nuclear membrane, escape degradation by endonucleases and, eventually, be expressed. Each of these steps represents a potential limitation to the efficacy of gene transfer, which make spontaneous transfer and expression of foreign DNA into eukaryotic cells a rare phenomenon. Transfer vectors are therefore required to increase the efficiency of the process. These may be viruses, non-viral vectors or mixed systems combining viral and non-viral elements.

2.1. Viral vectors

Viral-based vectors are viral particles which retain their ability to enter target cells and transfer in these cells foreign genes, but have been engineered to incorporate the

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transgene in their genome and loose their replicative activity. Some of them (retroviruses, lentiviruses and possibly adeno-associated viruses) are the only methods ensuring stable integration of transferred DNA into the chromosomal DNA of the target cell. In order to transform viruses into safe vectors, genomic sequences which are required for viral replication have been deleted.

2.1.1. Retroviral vectors

These are single-stranded RNA viruses that bind to a specific cell surface receptor to get entry into the cell. Their capsid is surrounded by an envelope, the proteins of which mediate adhesion to the cell membrane. Following penetration into the cell, viral RNA is transformed into double-stranded DNA by the reverse transcriptase and the DNA integrates randomly into the host cell genome, creating a 'provirus'. The viral genome contains 3 structure genes, *gag*, *pol* and *env*, encoding the capsid proteins, the reverse transcriptase and the envelope proteins respectively. The genome also contains assembly sequences, including the Ψ gene, required for virion encapsidation. The retroviral vectors currently used [7] are derived from the Moloney murine leukemia virus. Schematically, in these vectors, the *gag*, *pol* and *env* genes have been deleted and replaced, by homologous recombination, by the transgene, while the Ψ sequence is retained. Due to packaging limitations, the size of the transgene in retroviral vectors is limited to 9 kb. In the absence of structure genes, these defective recombinant vectors are unable to replicate. In order to obtain viral stocks, it is therefore required to use 'packaging' cell lines, in which the *gag*, *pol* and *env* genes are stably expressed. Transfection of these cells by the defective retroviruses genome leads to transcomplementation of the retroviral genome and to the production of replication-defective retroviral vectors harboring the transgene.

Since the retroviral genome integrates into cell DNA, the transgene is transmitted to daughter cells and its expression remains stable. Therefore, retroviral vectors have been used extensively for gene transfer in general [8], and were the first vectors to be used for arterial gene transfer in particular [9–11]. Transfer is restricted to cells possessing the retroviral specific receptor. The receptors for both amphotropic, i.e., with extended tropism including human cells, and ecotropic, i.e., specific for murine cells, retroviruses have been cloned and are broadly expressed [12]. There are however, several problems associated with the use of retroviral vectors for arterial gene transfer: (1) retroviral vectors can only infect replicating cells [13], which represent only a few percents of vascular cells in normal or atherosclerotic arteries, even following balloon angioplasty [14,15]; (2) it is difficult to purify and concentrate these vectors in order to achieve the high titer solutions required for efficient gene transfer; (3) retroviral vectors are unstable and, therefore, inappropriate for in vivo gene transfer; (4) finally, integration of the retroviral genome into the host cell DNA carries potential risks of insertional mutagenesis as well as activation of cellular oncogenes following integration, which would argue for the restriction of retroviral vectors to the treatment of life-threatening diseases.

Retroviral vectors remain largely used in current gene therapy protocols, especially when the therapeutic gene is delivered ex vivo (indirect gene transfer) in proliferating cells which are subsequently transplanted back into the body [8]. However, they are poorly suited to in vivo gene transfer (direct gene transfer). Recent developments in the field of retroviral vectors include engineering of lentiviral vectors based on HIV, which have been shown to achieve stable in vivo gene transfer into non-dividing cells [16], as well as high titers pseudotyped vectors [17]. Whether these approaches will make retroviral vectors more effective and safe for clinical application however remains to be demonstrated.

2.1.2. Adenoviral vectors

Adenoviruses (for reviews, see Refs. [18–20]) are non-enveloped viruses carrying a 36 kb double-stranded DNA. There are nearly 50 adenoviral serotypes, but only serotypes 2 and 5 have been used for gene transfer. Adenoviral genome is composed of regions which are expressed 'early' (E1–E4) or 'late' (L1–L5) relative to viral DNA replication. The expression of adenoviral genes is controlled by cellular transcription factors and by the E1 region which encodes a transactivating factor. Adenoviruses enter the cell through a receptor-mediated endocytosis pathway. Adenoviral particles bind to two receptor-types, including a glycoproteic receptor specific for the adenovirus fiber protein and surface integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) that serve as receptors for the adenovirus penton protein, and are then internalized by endocytosis. Acidification of the endosomal content results in conformational changes of the viral capsid proteins, which leads to rupture of the endosomal vesicle and liberation of the viral DNA in the cytoplasm, before DNA degradation by lysosomal enzymes. Viral DNA is then transported from the cytoplasm into the transduced cell nucleus, where it remains episomal. Endosomolysis is a key feature of adenoviruses which is largely responsible for the high transfection efficiencies reported with adenoviral vectors in numerous organs [21–26].

First generation recombinant adenoviral vectors currently used (for a review, see [27]) are obtained through homologous recombination between the genome of a serotype 5 or 2 adenovirus, which has been deleted of its leftward part, and a shuttle plasmid in which the transgene has been inserted along with the leftward part of the adenoviral genome, in order to facilitate recombination (Fig. 1). Expression of the transgene is usually driven by a 'strong' viral promoter, such as the Rous sarcoma virus long terminal repeat (RSV LTR), the cytomegalovirus (CMV) immediate-early promoter/enhancer, or the major late promoter and tripartite leader of adenovirus 2 or 5.

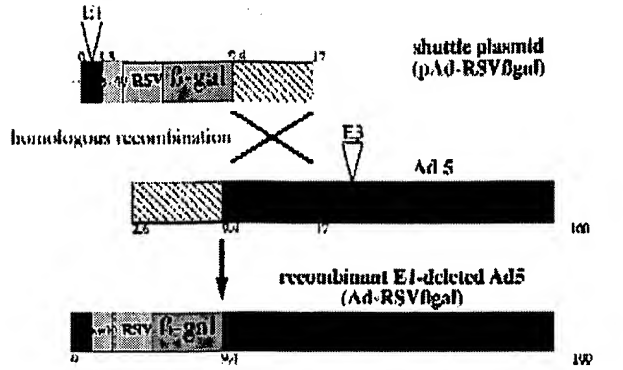

Fig. 1. Construction of recombinant, replication-defective (E1-deleted) adenoviral vectors. The genome of recombinant adenoviral vectors is obtained through homologous recombination between (1) a shuttle plasmid (pAd-RSVβgal) carrying the left end (map units 0 to 17) of the Ad5 genome, except E1 which is replaced by a nuclear localization signal (SV40), the Rous Sarcoma Virus long terminal repeat (LTR) and the β-galactosidase reporter gene. and (2) the rightward part of the Ad5 genome (map units 26 to 100), in which the E3 region has been deleted.

These are constitutive promoters, which therefore lead to synthesis of large amounts of recombinant protein, but lack tissue specificity and allow no regulation once the gene is transferred. The vectors are made replication-defective by deletion of the E1A and E1B sequences from the viral genome. The E3 sequence is also deleted in order to accommodate insertion of long transgenes (up to 7.5 kb). Homologous recombination as well as propagation of recombinant adenoviral vectors are achieved by co-transfection in 293 cells, a complementing cell line which constitutively expresses the E1 gene.

Adenoviral vectors allow to circumvent some of the problems encountered with retroviruses: (1) they can infect quiescent as well as replicating cells; (2) high titer stocks can be produced easily, generally ranging from 10^{11} to 10^{12} plaque forming units (pfu)/ml; (3) transfection efficiencies of 100%, for endothelial cells [28] and ~ 5%, for medial smooth muscle cells [29,30], have been recently reported in animal models of arterial gene transfer; and (4) after infection, the adenovirus genome remains episomal, therefore avoiding the risk of insertional mutagenesis.

Nevertheless, several drawbacks of adenoviral vectors have been identified. First, current adenovectors are associated with only transient, 2- to 4-week, transgene expression [31]. Second, at a high multiplicity of infection (MOI) — i.e., the ratio of infectant viral particles to target cells — replication may be observed in E1-deleted adenoviral particles. This may be related to the presence of proteins with E1A-like activity in host cells [32]. Alternatively, replication competent adenovirus (RCA) may emerge during production of E1-deleted viral stocks through homologous recombination between the transgene and the E1 region of 293 cells. Third, it has been demonstrated that first-generation adenovectors evoke a strong cellular immune response, targeted at viral proteins as well as certain transgene products [33,34], resulting in destruction of these cells by cytotoxic T lymphocytes [35]. In addition, infection of the target cells by adenoviral vectors leads to an acute inflammatory reaction, characterized by a neutrophil- [36] and macrophage-rich [37] cellular infiltrate. Finally, it has been observed that local delivery of adenoviral vectors in normal arteries upregulates the expression of vascular cell adhesion molecules and may even trigger mild, although significant, neointima formation [38]. The respective roles of the vector, the transgene, RCAs or impurities related to vector processing, in the generation of adverse effects associated with first-generation adenoviral vectors remain unclear.

The main consequences of the immune/inflammatory reaction directed against recombinant adenoviral vectors are rapid extinction of transgene expression (within a few weeks) and the occurrence of inflammatory reactions in the recipient. Repeated injections of adenoviral vectors might not result in prolonged transgene expression [39] due to a humoral immune response targeted at both viral proteins and transgene products [40]. Interestingly, the duration of transgene expression is substantially prolonged when transfer is achieved in newborn animals [41], probably because these are not yet immunocompetent and can therefore tolerate viral proteins, or in animals in which cellular immune response has been pharmacologically depressed [42]. Immunosuppression, however, cannot be used routinely in clinical practice. Thus, new adenoviral vectors, termed second and third-generation vectors, are currently developed [32,43,44]. These vectors are modified to prevent in a more efficient fashion residual expression of adenoviral proteins, thereby mitigating adenoviral protein-specific cellular immune response, which in turn leads to less inflammatory reaction as well as protracted transgene expression. It must be borne in mind, however, that transgene expression is transient not only because of the cellular immune response, but also because of the episomal situation of the transgene, 'extinction' of promoter sequences and because of transcriptional and post-transcriptional mechanisms which remain largely unknown. In certain indications, however, such as prevention of restenosis, transient gene expression may be sufficient and may even confer a relative safety to adenoviral vector-based strategies for gene therapy, since potential deleterious effects related to transgene expression would be limited to a few weeks.

2.1.3. Other viral vectors

Other viral vectors are being developed for gene transfer but have not been tested in models of arterial transfection. The AAV (adeno-associated viruses) are single-stranded DNA parvoviruses. Wild-type AAV have the ability to integrate stably their genome into chromosome 19q13 of the human cellular genome. Such 'targeted' integration could theoretically limit the risk of insertional mutagenesis related to random integration into the host cell

genome. However, recombinant AAV [45] do not appear to exhibit the same site specific integration as wild-type virus. Alternatively, herpes viruses would result in protracted transgene expression. They are however, toxic to the transfected cell, even in their replication-defective form [8].

2.2. Non viral vectors

The limitations and risks associated with viral vectors are powerful stimulants for the development of non viral vectors. DNA delivered by nonviral methods is maintained in an extra-chromosomal state and is not integrated into the cellular genome [46].

2.2.1. Naked DNA

It is possible to incubate plasmid DNA with cells and obtain non-specific DNA uptake, which generally does not result in DNA integration into the host cell genome. When integration occurs, a rare phenomenon, it is random and carries a risk of insertional mutagenesis. Although this technique has a low efficiency of transfer, it is simple and safe. It is extremely well suited to ex-vivo transfer when transduced cells can be sorted using a selectable marker gene, but it has also been used clinically [47,48].

2.2.2. Cationic liposomes

Cationic liposomes are positively charged artificial lipid vesicles, which incorporate negatively-charged plasmid DNA. In contrast with viral vectors, there is no size constraint for the transgene and preparation of the vectors is easy (Fig. 2). DNA-liposomes complexes contain an excess of liposomes and therefore of positive charges, which facilitate fusion between liposomes and the negatively-charged cell membranes [49]. Once in the cytoplasm, most of the DNA-liposome complexes are degraded by lysosomal enzymes, and approximately 1% of the DNA which originally penetrated the cell enter the nucleus where it remains extra-chromosomal. This explains why transgene expression is transient when liposomes are used as vectors. Various cationic liposome preparations are commercially available: DOTMA-DOPE (Lipofectin), DOSPA/DOPE (Lipofectamine), DC-cholesterol, DMRIE/DOPE. The efficiency of cationic liposomes for gene transfer is superior to that of other non-viral methods and varies with the preparation used, the DNA/liposome concentration ratio, the type and the proliferative status of the transfected cells (transfer appears increased in proliferating cells). While in theory, under optimal conditions, in vitro transfection efficiencies can reach up to 90% of target cells, experience with in vivo arterial gene transfer suggests that efficiency remains disturbingly low [50–52]. Compared to viral vectors these vectors are at least 3 logs less efficient, and require extremely high DNA concentrations to achieve successful gene transfer. Finally, cationic liposomes are potentially toxic due to the cellular accumulation of lipids. This untoward effect, however, has not been observed in several studies in which DNA/liposomes complexes were introduced systemically [53].

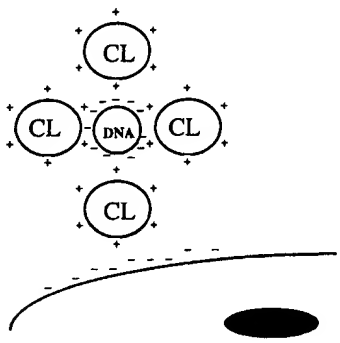

Fig. 2. Schematic representation of cationic liposomes. Positively charged cationic liposomes are complexed with negatively charged DNA, and penetrate into the cell via membrane fusion, facilitated by the electrostatic interaction between the positive liposome charges and the negative cell membrane charges.

2.2.3. Conjugated vectors

Conjugated vectors (for a review, see [54]) represent a heterogeneous class of vectors in which the transgene is conjugated to a polycation (e.g., polylysine) which is chemically bound to a proteic ligand. Polylysine forms a complex with negatively-charged DNA via electrostatic interaction, and condenses the DNA into a macromolecule-like structure. Binding of the ligand to its specific membrane receptor mediates vector internalization into the cell. Several studies have established that selective in vitro gene transfer can be achieved using this method. In these studies, the ligand was either an asialoglycoprotein, which is recognized by hepatocytes, or transferrin, which is recognized by many replicating cell lines, or polylysine-bound osidic residues. The main limit of this method is its poor efficiency, related to transgene degradation by lysosomal enzymes. Several methods have been used to improve somewhat transfection efficiency, such as addition of chloroquine (which prevents acidification of the endosomal content, thereby preventing transgene hydrolysis) or fusion peptides (which disrupt the endosome membrane and release the transgene into the cytoplasm). Such conjugated vectors have been used in vivo to obtain transient and partial correction of the hypercholesterolemia of Watanabe rabbits via transfer into hepatocytes of the gene encoding for the LDL receptor [55].

2.3. Mixed vectors (viral / non viral)

These vectors are designed to combine the benefits of both viral and non viral vectors.

2.3.1. Conjugated vectors associated with defective adenoviruses

The combination of replication-defective adenoviruses and conjugated vectors is designed to increase gene trans-

fer efficiency by using the adenovirus capability for endosomolysis, while retaining the specificity of transfer associated with ligand/membrane receptor interactions. Several methods have been used. The most simple consists in incubating the target cells in the presence of a mixture of conjugated vectors (e.g., DNA-polylysine-transferrin) and defective adenoviruses. This method is 2000-fold more efficient than that using conjugated vectors alone [56]. Efficiency is even greater when defective adenoviruses are coupled to conjugated vectors via a monoclonal antibody targeted at the hexon protein of the adenoviral capsid [57]. In this case, however, transfer specificity is lost due to the interaction between the adenoviral fiber protein and its specific receptor. This problem has been solved by masking the fiber protein, either by a monoclonal antibody, or by oxidation of the fiber protein. These mixed conjugated vectors are very efficient in vitro. In contrast with recombinant adenoviral vectors, the size of the transgene is not a limiting factor, since it is not incorporated into the viral genome. However, in vivo efficiency of these vectors is limited by their instability and the requirement to cross the endothelial barrier in case of systemic injection. For this reason, mixed conjugated vectors are used in models of ex vivo (indirect) gene transfer [58]. Even under these 'optimal' conditions, duration of transgene expression is limited to approximately 2 weeks, probably because of non-integration of the transgene into the host cell genome and viral protein-specific immune response.

2.3.2. Conjugated vectors associated with the Hemagglutinating Virus of Japan (HVJ)

These vectors integrate liposomes, DNA and UV-inactivated HVJ particles. This combination has been proved effective in models of liver and kidney transfection, probably due to the ability of HVJ virus particles to penetrate the cell. It is assumed that when one HVJ particle gains access to the cell, via a specific receptor-mediated pathway, one or several liposome/DNA complexes are internalized in the cell and a certain amount of internalized DNA reaches the nucleus where it is expressed. The absence of toxicity of these vectors and their efficiency (~ 10-fold that of cationic liposomes) for vascular smooth muscle cell transfection, make them an attractive tool for arterial gene transfer [59]. These vectors have been used for a host of cardiovascular applications (for a review, see [60]), including the study of the effect of autocrine-paracrine vasoactive modulators (e.g., the renin-angiotensin system) on vascular smooth muscle cells in vitro [61] as well as the inhibition of intimal hyperplasia using nitric oxide synthase cDNA [62], antisense oligonucleotides directed towards cell-cycle positive regulators [63], or transcription factor 'decoys' [64].

3. Gene delivery techniques

3.1. Percutaneous local delivery systems

Most potential targets of arterial gene therapy would require local expression of the transgene at a specific arterial site. This can be achieved either by delivering the gene vector in the vicinity of the target site, i.e., local delivery, or by incorporating in the vector design a ligand which will drive transgenic expression at that site, even if the vector is injected systemically. Advances in local gene delivery to the arterial wall have largely benefited from progress made in the technology of angioplasty balloon catheters. The simplest method for local arterial gene transfer is the 'dwell method', which requires to isolate surgically between two temporary ligatures an arterial segment, withdraw blood, inject into the isolated segment a solution containing the vector, then withdraw this solution after a variable incubation time and reestablish blood flow [28,65,66]. Dwelling allows to control several of the parameters which determine gene transfer efficiency: absence of leakage from the transfer compartment, minimization of vector loss in the systemic circulation, shortened transfer handling time, ..., etc. The invasive nature of the

Table 1
Currently available local delivery catheters

Device	Mechanism	Target cells	Released for clinical use
Double-balloon (USCI)	Diffusion	Endothelium	No
Dispatch (Scimed)	Diffusion	Endothelium	Yes
Hydrogel balloon (Boston Sc.)	Diffusion + pressure	SMC	Yes
Coated stents (Johnson&Johnson)	Diffusion + apposition	SMC	Yes
Porous balloon (USCI)	High pressure	SMC, adventitia	No
Microporous balloon (Cordis)	Low pressure	SMC?	No
Channelled balloon (Boston Sc.)	Low pressure	SMC	Yes
Transport (Scimed)	Low pressure	SMC?	Yes
InfusaSleeve (Localmed)	Low pressure	SMC?	Yes
Iontophoretic balloon (ēMed)	Electric gradient	SMC?, adventitia?	No
Needle balloon	Puncture	SMC? adventitia?	No
Nipple balloon (interventional ther.)	Puncture	SMC? adventitia?	No

Putative mechanisms of gene delivery are mentioned as well as main target cell-types.
smc, smooth muscle cell.

method, however, is a major drawback for clinical application. For this reason, various local gene delivery systems have been developed (for reviews, see [67,68]).

3.1.1. Catheters

An 'ideal' local delivery catheter should incorporate the following features to perform optimal arterial gene transfer. First and foremost it should achieve very efficient gene transfer. Second, if it is intended to be used for prevention of restenosis, balloon angioplasty and gene transfer should be performed simultaneously during a one-step straightforward procedure using the same catheter. Third, the local delivery device should be safe, and, in particular, should not induce excessive injury to the arterial wall, in addition to the injury associated with the angioplasty per se. Fourth, gene delivery catheters should incorporate a perfusion design to limit myocardial ischemia during gene incubation. Finally, site-specificity is a key issue for arterial gene therapy. Local delivery catheters should be designed in order to minimize gene leakage in the bloodstream, which may limit local efficacy and result in systemic toxicity, especially when viral vectors are required.

Currently available local delivery catheters are listed in Table 1. It must be stressed, however, that the vast majority of them have only been tested for arterial delivery of pharmacologic agents to normal arteries. Therefore, data obtained from the literature may not be directly applicable to gene delivery, in particular in atherosclerotic arteries. Schematically, local delivery through catheters involves 3 basic, device-related mechanisms: passive diffusion, pressure facilitation, and mechanical facilitation.

3.1.1.1. Passive diffusion. The double balloon catheter is made of two latex balloons which, when inflated into the target arterial segment, delineate a 'transfection chamber' of varying length (usually 15 to 20 mm), into which the transfer vector can be instilled via an infusion port, generally using pressure. A retrieval port is usually available to withdraw the solution at the end of the incubation period. This catheter was the first to be used for catheter-based arterial gene transfer [10]. It has, however, several shortcomings. Passive diffusion of the gene vector requires long incubation times, which may generate tissue ischemia. There is a risk of vector diffusion into the systemic circulation via side branches (such side branches emerge every 2–4 mm in the epicardial coronary arteries), which may result in both decreased transfer efficiency and viral dissemination. Finally, inflation of the two latex balloons is a source of additional arterial trauma both upstream and downstream of the transfected segment.

New catheters represent 'improved' designs of the double-balloon catheter. The Dispatch™ catheter is a sophisticated catheter allowing simultaneous distal perfusion and isolation of multiple infusion chambers between the catheter and the vessel wall [69]. The main advantage of this device is that even protracted incubations do not induce significant tissue ischemia [70]. This system has been successfully used to achieve substantial gene delivery into the endothelium and superficial medial layers of both normal and atherosclerotic rabbit arteries [71]. However, the infusion chambers do not ensure total isolation from the systemic circulation; therefore the Dispatch™ catheter, like most other local delivery catheters, is associated with a substantial risk of vector dissemination, in particular in the liver.

The hydrogel-coated balloon catheter is a conventional angioplasty balloon which is coated with a hydrophilic polymer which swells like a sponge in presence of a solution containing a drug or a gene vector. Upon inflation of the angioplasty balloon in the target vessel, the polymer express the adsorbed materials toward the arterial wall [72]. This balloon was initially designed to cross high-grade complex lesions. It turned out to be a very efficient local delivery system which has been released for clinical use in Europe and United States. This catheter is currently used for intracoronary delivery of urokinase during primary angioplasty for acute myocardial infarction [73], and for local delivery of the VEGF cDNA in the peripheral arteries of patients suffering end-stage arteriopathy [48]. Its main shortcoming is that during exposure of the catheter in the bloodstream, most of the hydrogel content is 'washed' off the balloon [69]. However, retention into the polymer may be enhanced by using a protective sheath.

3.1.1.2. Pressure facilitation. The Wolinsky catheter incorporates an angioplasty balloon with 25 μm diameter pores. During balloon angioplasty, inflation pressure drives the solution filling the balloon into the arterial wall [74]. However, at high inflation pressures typically required for optimal angioplasty (5 to 10 atm), porous balloons generate high velocity jets, which result in arterial perforation as well as reactive intimal hyperplasia, and carries a risk of gene dissemination. Therefore, improved porous balloon catheters have been designed to overcome these limitations, principally to dissociate balloon inflation pressure from vector instillation pressure.

The microporous balloon catheter [75] incorporates an internal porous balloon, with 25 μm diameter pores, and a second external membrane with thousands of micropores of less than 1 μm diameter. These micropores tend to limit jetting and wall trauma.

The channeled balloon catheter (Fig. 3) is a conventional angioplasty balloon covered with 24 longitudinal channels, each having a 100 μm diameter pore perfused via a separated lumen, allowing local low pressure instillation during high pressure balloon inflation [76].

3.1.1.3. Mechanical facilitation. The mechanism used to facilitate local delivery may be either an electrical field, in he case of the iontophoretic catheter, or a physical injury to the vessel wall in the case of the needle catheter or the nipple balloon. The iontophoretic balloon is a porous

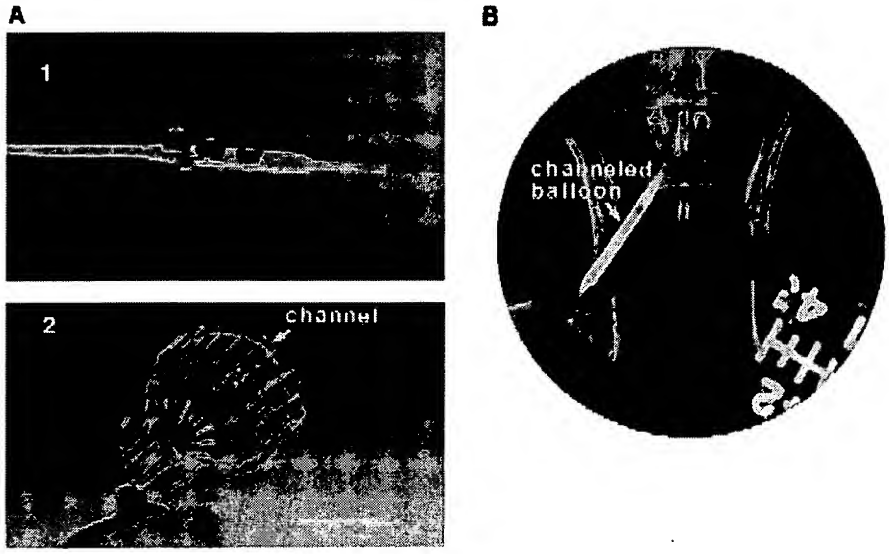

Fig. 3. The channelled-balloon catheter. (A) Longitudinal (1) and cross-sectional (2) views of the channelled-balloon. Gene vector solutions are infused through small perforated channels which are located on the surface of the angioplasty balloon. Pressures for balloon inflation and vector infusion can thus be separated. (B) Radiograph showing the channelled-balloon inflated in a rabbit external iliac artery. Balloon-angioplasty and transfection are performed simultaneously. Infusion of contrast medium into the balloon inflation lumen delineates balloon contour.

balloon with an inner electrode which serves as a cathode. An anode is applied on the skin. Electric current drives negatively-charged molecules outside of the balloon into the arterial wall [77]. This system appears particularly well suited to deliver negatively-charged plasmid DNA in the arterial wall.

3.1.2. Intracoronary stents

Clinical use of metallic intracoronary stents has become standard practice during routine PTCA to treat established coronary dissection. Intracoronary stents such as the Palmaz–Schatz stent have led to a reduction in the incidence of restenosis compared to conventional balloon angioplasty, most likely due to a reduction of acute and chronic constrictive remodeling of the vessel wall as well as an optimization of the initial geometric result of PTCA [78,79].

In addition, intracoronary stents may be used as vehicles for local delivery of drugs or gene vectors into the arterial wall (for reviews see [68,80]). One of the approaches would be to coat the stent struts with endothelial cells, previously transfected with a therapeutic gene, the product of which may be released locally to exert therapeutic effects. In vitro experiments with metallic stents seeded with normal [81], immortalized [82], or tPA-transfected [83,84] endothelial cells have established the feasibility of this technique. However, clinical applicability of this technique remains limited due to the requirement of previous isolation and in vitro transfection of endothelial cells — a long and costly procedure — as well as the poor adhesion of these cells to the stent struts following expansion under flow condition. Another strategy is to use polymer-coated stents, which may act as a reservoir for a gene vector [85], or even biodegradable polymeric stents [86].

3.1.3. Polymers

The 'ideal' polymer for arterial gene transfer should contain large concentrations of genetic materials to ensure local delivery and protracted residence of the latter into the arterial wall. It should also be biocompatible for the blood/wall interface, and, if possible, biodegradable. Biodegradable polymers could be used for arterial gene transfer in the form of intracoronary stents, micro- or nano-particles injected locally via a catheter [87], gels ('pavement') coating the endoluminal aspect of the artery [88], or periadventitial wrapping [89]. The latter method has been used to transfer plasmidic DNA [90] as well as antisense oligonucleotides [91] into the rat carotid artery, in order to prevent intimal thickening following arterial injury. In addition, we recently demonstrated that co-delivery of adenoviral vectors together with the block copolymer poloxamer 407 facilitates arterial transfection and allows for shorter incubation times [37].

3.2. Myocardial delivery

Pioneered by Wolff et al. [92], intramuscular gene transfer represents an alternative method to target the myocardium. Direct injection of plasmid DNA into the myocardium, although feasible [93–95], has been associated with short-lived (2 to 4 weeks) expression of the transgene in only a small number of cells, as well as with potentially deleterious myocardial fibrosis and inflammation. When intravenous injection of recombinant replication defective adenoviral vectors is used, only minor myocardial uptake is observed [41]. In contrast, direct injection of such vectors in the heart resulted in efficient

transfection [26,96], albeit limited by the previously described shortcomings associated with adenoviral vectors (transient expression of the transgene, immune and inflammatory responses) and by the need for direct access to the myocardium which requires intraoperative, pericardial or perimyocardial injections. Alternatively, at least three studies have reported efficient transfection of the myocardium after intracoronary catheter delivery of adenoviral vectors [97–99]. Pressure conditions during delivery as well as pretreatment of the vessel with various vasoactive agents may impact heavily on the feasibility of myocardial gene transfer via the intracoronary route [99].

4. Arterial gene transfer: Feasibility and problems

Two strategies can be used for gene transfer: indirect gene transfer involves in vitro transfection of vascular cells which are then implanted back into the vasculature; conversely, direct gene transfer is the direct introduction of a foreign gene into the arterial wall in vivo (Fig. 4).

4.1. Indirect gene transfer

In their seminal experiments, Nabel et al. used recombinant retroviral vectors to transfer the β-galactosidase reporter gene into porcine endothelial cells in vitro [9]. After staining with the specific chromagen X-gal, transfected endothelial cells could be easily recognized and selected for subsequent introduction into the iliac artery in vivo using a double-balloon catheter. Expression of the transgene was found in these arteries several weeks after transfer. A similar strategy was successfully applied to in vitro transfected smooth muscle cells [11]. Other techniques for indirect gene transfer were later suggested such as seeding of genetically modified endothelial cells on the surface of endovascular metallic stents [83,84], of Dacron arterial prosthetic grafts [100], or of venous grafts [101]. Indirect gene transfer allows for in vitro selection of successfully transduced cells prior to arterial delivery. However, it is only suitable for autologous transfection, i.e., transfected cells can be transplanted only in the same patient from who these cells have been harvested. Moreover, it requires cell isolation and culture prior to transfer, which makes it a cumbersome and costly technique for routine clinical application [5,8], with the exception of ex vivo transfer into saphenous vein grafts during coronary artery bypass surgery, for which the tissue to be transfected is readily available for ex vivo transfer.

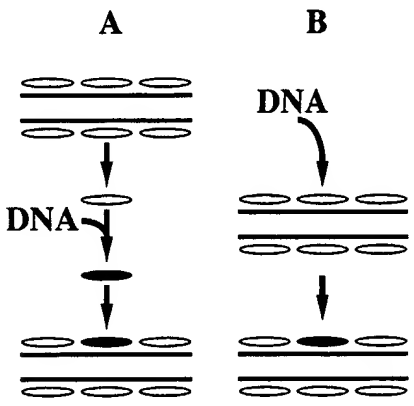

Fig. 4. Direct vs. indirect gene transfer. (A) Indirect gene transfer: gene transfer is performed in vitro, and the transfected cells are subsequently reimplanted in the target organ. (B) Direct gene transfer: the transgene is directly introduced into the target organ in vivo via systemic or local (surgical or catheter-based) delivery.

4.2. Direct gene transfer

Direct gene transfer is a one-step method in which the transgene incorporated in a vector is directly delivered into the target arterial site. Again, Nabel et al. were the first to achieve direct arterial gene transfer using replication-defective retroviral vectors and cationic liposomes expressing the LacZ reporter gene, introduced via a double balloon catheter into porcine iliac arteries [10]. β-galactosidase activity was observed in all the transfected animals, up to 21 weeks in those animals transfected using retroviral vectors and 6 weeks when liposomes were used. Other studies using retroviral vectors [102], cationic liposomes [50–52] or plasmid DNA [103,104], have confirmed these results. However, in all theses studies transfection efficiency was far below 0.1% when nuclear-specific β-galactosidase was used as reporter gene (allowing discrimination from endogenous cytoplasmic β-galactosidase activity).

Low transfection efficiency is a major limitation of these methods, in particular when the transgene encodes a protein which remains intracellular, a frequent feature for many of the candidate genes used in attempts to prevent restenosis [6]. Conversely, when the transgene encodes a secreted protein, such as the growth hormone [105] or a secreted growth factor [106,107], a substantial biological effect may be observed even when a small number of target cells express the transgene.

Replication-defective adenoviral vectors have been used for gene transfer into the arterial wall, using surgical [28,36,37,65,66,108–111] or percutaneous [29,30,112–116] transfer techniques. The first generation vectors used in these experiments were replication defective due to deletion of the E1 sequence. When such a vector expressing nuclear-targeted β-galactosidase is delivered in contact with the endothelial layer without previous injury, for example using a surgical 'dwell' method or a double balloon catheter, transfer is strictly localized to the endothelium (Fig. 5) [29,111,115]. In addition, as previously mentioned, expression of the transgene is confined to the first days or weeks following delivery. Transfection efficiency is related to the concentration of the adenovirus

stocks used [111]: efficiency increases with viral concentration when the latter remains $\leq 5 \cdot 10^{10}$ pfu/ml; it then reaches a plateau, related to direct cytotoxicity of the vector. The magnitude and mechanism of this cytotoxic effect appear to vary with vector design and preparation. When concentration is further increased beyond 10^{11} pfu/ml, efficiency decreases. Under certain conditions, however, transfection efficiency approximates 100% [28,29]. In intact vessels, the endothelium is therefore the elective target of replication-defective adenoviral vectors.

When adenovirus-mediated gene transfer is attempted following abrasion of the endothelial layer, transgene expression is found mostly in smooth muscle cells located in the superficial layers of the media (Fig. 6) [29,30,36,37,66,110,112]. Reported transfection efficiencies, expressed as the percentage of transfected medial cells, range from 2 to 70%. Adenoviral vectors are thus, by far, the most effective vectors for medial transfection. Transfection efficiency in the media depends upon various factors, including virus preparation (concentration of viral stocks, promoter sequences), delivery technique (dwell > catheter-based), catheter-type (hydrogel balloon > double balloon), duration of incubation (long > short), ligation of side-branches, as well as co- or pre-treatment with adjunctive agents. The endothelium and internal elastic lamina are the main barriers to penetration of the adenoviral vectors in the media of non atherosclerotic vessels [29,115,117], whereas the neointima is a relatively resistant layer to adenovirus penetration in severely atherosclerotic arteries [30,118]. Recently, Perlman et al. demonstrated that balloon angioplasty rapidly induces massive apoptosis in the arterial wall, mostly in the superficial layers of the media [119], an anatomic feature which may influence transfection efficiency as well. Low transfection efficiency in atherosclerotic vessels must be considered when potential clinical applications relate to atheromatous arteries. Methods to circumvent low transfer efficiency include the use of therapeutic genes encoding for secreted proteins, which may therefore affect not only transduced but also neighboring untransduced cells [6]. Alternatively, transfection efficiency can be enhanced by using adjunctive agents such as elastase, to permeate the internal elastic lamina [117], or polymers such as poloxamer 407 [37,120].

Fig. 5. Endothelium-specific arterial gene transfer. An adenoviral vector expressing a nuclear-specific β-galactosidase reporter gene was locally delivered in rat carotid arteries using the dwell technique. Three days following gene transfer, β-galactosidase activity is found exclusively in the endothelial layer (dark nuclei). L, lumen; M, media; A, adventitia. (Feldman et al., unpublished data.)

Fig. 6. Smooth-muscle cell-specific arterial gene transfer. Percutaneous gene transfer was performed in rabbit iliac arteries. An adenoviral vector expressing the nuclear-specific β-galactosidase reporter gene was locally delivered using a channelled-balloon catheter. In this case, balloon angioplasty and gene transfer are performed simultaneously. Three days following gene transfer, transduced cells (dark nuclei) are located in the superficial as well as deep layers of the media. These cells are easily identified as vascular smooth muscle cells. Note that the endothelial layer has been totally abraded, whereas the internal elastic lamina appears well preserved. L, lumen; M, media; A, adventitia. (Feldman et al., unpublished data.)

The delivery device used has also a major impact on the risk of systemic dissemination of viral particles, which is high in the case of amphotropic adenoviral vectors. This may be explored by studying expression of the transgene, or rather its presence (by PCR) in organs remote from the transfer site. Pervious studies have shown that the risk is low when adenoviral vectors are introduced into a surgically isolated arterial segment [28,37]. When percutaneous delivery is attempted, however, the risk of viral dissemination becomes substantial, most of extra-arterial transfection being located in the liver [29,37]. The risk is high with the double balloon catheter [29], the porous balloon catheter [121], or the dispatch catheter [71]. It is reduced with the channeled balloon catheter [30], and appears low with hydrogel balloon catheters, at least when a protective sheath is used [29]. The risk is also correlated to the degree of arterial trauma induced by the gene delivery device [114]. Other factors may promote viral spreading such as the use of non-specific viral promoters, high viral titers, as well as the presence of developed vasa vasora in the arterial wall, a typical feature of the atherosclerotic plaque.

Another issue is that in most of currently available transfection systems transgene expression cannot be regu-

lated by physiological or exogeneous signals, which may represent an important limitation for clinical application of gene therapy to some diseases in which precise control over the level of protein production is required to achieve a therapeutic effect and/or prevent toxicity. Several approaches have been tested in transgenic animals and in models of somatic gene transfer to regulate transgene expression [122–124]. The basic element of any system is a pharmacologic agent that modifies the activity of a transcription factor, which is capable of regulating a heterologous promoter that drives transgene expression. For exemple, Bohl et al. have recently designed a system in which two genes packaged into distinct retroviral vectors are transferred in primary myoblasts in vitro: an erythropoietin cDNA driven by a tetO-CMV promoter and a reverse transactivator (rtTA). When doxycycline is added, rtTA binds the tetO-CMV promoter and activates transcription of the erythropoietin cDNA [124]. To date, none of these systems have been tested in models of cardiovascular gene transfer. However, tight regulation of transgene expression may not be as crucial in cardiology as in inheritable metabolic diseases (see below).

5. Conclusions and perspectives of clinical application

In vivo transfection of foreign genes in the cardiovascular system has broad applications. Retroviral vectors have been used to study cardivoascular development [125] and to learn more on the role of growth factors in the pathophysiology of intimal hyperplasia [106]. Clinical use of transfection techniques to treat cardiovascular disease represents a more challenging issue [5].In vivo application of gene therapy requires a unique combination of appropriate vector, delivery system, target cell and therapeutic gene which is likely to be specifically tailored to each application. Therefore, there is no 'best' vector or delivery system. In fact, efforts should be focused on designing the appropriate combination required to approach each clinical situation, which should be tested in the appropriate experimental models [126,127]. Current indications for arterial gene therapy should fulfill the following requirements: (1) it should be a disease without proven efficient conventional therapy (due to either ineffectiveness or major adverse effects); (2) the arterial lesions to be treated should be amenable to local delivery techniques; (3) pathophysiology should be clear enough for identification of candidate therapeutic genes.

There are currently two major potential indications for arterial gene therapy: prevention of restenosis after angioplasty and therapeutic angiogenesis to treat chronic ischemia, either in the myocardium or in the limbs (for reviews, see [5,6,47,128]). Other potential indications are prevention of degeneration of aortocoronary saphenous vein bypass grafts [101] and atheromatous plaque stabilization [129], but nearly all vascular diseases are theoretically amenable to gene therapy [4,130], as well as a host of non vascular diseases in which arterial access to the diseased organs is available for local genetic treatment.

Genetic prevention of restenosis best exemplifies the importance of gene delivery techniques in achieving therapeutic success. Indeed, it has long been considered that restenosis — i.e., the recurrence of luminal narrowing in the months following angioplasty — results almost exclusively from intimal hyperplasia, a proliferating process involving medial and intimal layers of vascular smooth muscle cells [131]. Therefore, most of current strategies aimed at preventing restenosis by gene therapy consist in local transluminal delivery of antiproliferative genes in the abluminal arterial smooth muscle cells in order to inhibit intimal hyperplasia [5,128]. However, recent advances in the understanding of the pathophysiology of restenosis [132] indicate that, both in experimental models [133] and in humans [134], chronic constrictive remodeling (or the absence of compensatory vessel enlargement) plays a major role in late luminal loss and that lumen diameter is strongly correlated to the magnitude of constriction but not to intimal thickness. The mechanism of constrictive remodeling, although still unclear [135], may involve biological changes in the adventitia including inflammation, myocellular proliferation and migration, and fibrosis [136–138]. Should constrictive remodeling become a target for antiproliferative gene therapy, maybe periadventitial gene delivery will be more appropriate than current intraluminal delivery techniques. Adventitial transfection, however, is likely to induce unacceptable arterial trauma which may outweigh the potential benefit of the therapy. Alternatively, endovascular stents are both efficient and safe to prevent arterial constriction. It has been convincingly demonstrated that in-stent restenosis, which occurs in roughly 25% of the patients [78,79], is almost exclusively composed of intimal hyperplasia [139]. Based on these recent data, the concept of integrated strategies to prevent restenosis arose, in which anti-remodeling stents are combined with antiproliferative genes to achieve optimal prevention of restenosis [140]. If integrated strategies are to be applied to the prevention of restenosis, we should consider developing in-stent gene delivery techniques as a priority. In this regard, preliminary results from a study by Van Belle et al. suggesting that efficient in-stent transfection can be performed percutaneously are encouraging [141].

References

[1] Cooper ES. Prevention: The key to progress. Circulation 1993;24:629–632.
[2] Ross R. The pathogenesis of atherosclerosis: A perspective for the 1990s. Nature 1993;362:801–809.
[3] Dzau VJ, Gibbons GH, Cooke JP, Omoigui N. Vascular biology and medicine in the 1990s: Scope, concepts, potentials, and perspectives. Circulation 1993;87:705–719.
[4] Gibbons GH, Dzau VJ. Molecular therapies for vascular diseases. Science 1996;272:689–693.

[5] Nabel EG. Gene therapy for cardiovascular disease. Circulation 1995;91:541–548.
[6] Isner JM, Feldman LJ. Gene therapy for arterial disease. Lancet 1994;344:1653–1654.
[7] Danos O, Mulligan RC. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. Proc Natl Acad Sci USA 1988;85:6460–6464.
[8] Mulligan RC. The basic science of gene therapy. Science 1993;260:926–932.
[9] Nabel EJ, Plautz G, Boyce DM, Stanley JC, Nabel GJ. Recombinant gene expression in vivo within endothelial cells of the arterial wall. Science 1989;244:1342–1344.
[10] Nabel EG, Plautz G, Nabel GJ. Site-specific gene expression in vivo by direct gene transfer into the arterial wall. Science 1990;249:1285–1288.
[11] Plautz G, Nabel EG, Nabel GJ. Introduction of vascular smooth muscle cells expressing recombinant genes in vivo. Circulation 1991;83:578–583.
[12] Weiss R, Taylor C. Retrovirus receptors. Cell 1995;82:531–533.
[13] Miller DG, Adam MA, Miller AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol Cell Biol 1990;10:4239–4242.
[14] O'Brien ER, Alpers CE, Stewart DK, et al. Proliferation in primary and restenotic coronary atherectomy tissue: Implications for antiproliferative therapy. Circ Res 1993;73:223–231.
[15] Pickering JG, Weir L, Jekanowski J, Kearney M, Isner JM. Proliferative activity in peripheral and coronary atherosclerotic plaque among patients undergoing percutaneous revascularization. J Clin Invest 1993;91:1469–1480.
[16] Naldini L, Blömer U, Gallay P, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 1996;272:263–267.
[17] Friedman T, Yee J-K. Pseudotyped retroviral vectors for studies of human gene therapy. Nature Med 1995;1:275–277.
[18] Horwitz MS. Adenoviridae and their replication. In: Fields BN, Knipe DM, eds. Fundamental Virology. New-York: Raven Press, 1991:771-813.
[19] Graham FL, Prevec L. Adenovirus-based expression vectors and recombinant vaccines. In: Ellis RW, ed. Vaccines: New Approaches to Immunological Problems. Boston, MA: Butterworth-Heinemann, 1992:363-390.
[20] Wilson JM. Adenoviruses as gene-delivery vehicles. N Engl J Med 1996;334:1185–1187.
[21] Rosenfeld MA, Siegfried W, Yoshimura K, et al. Adenovirus-mediated transfer of a recombinant a1-antitrypsin gene to the lung epithelium in vivo. Science 1991;252:431–434.
[22] Jaffe HA, Danel C, Longenecker G, et al. Adenovirus-mediated in vivo gene transfer and expression in normal rat liver. Nature Genet 1992;1:372–378.
[23] Quantin B, Stratford-Perricaudet LD, Tajbakhsh S, Mandel J-L. Adenovirus as an expression vector in muscle cells in vivo. Proc Natl Acad Sci USA 1992;89:2581–2584.
[24] Le Gal La Salle G, Robert JJ, Berrard S, et al. An adenovirus vector for gene transfer into neurons and glia in the brain. Science 1993;259:988–990.
[25] Ragot T, Vincent N, Chafey P, et al. Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of mdx mice. Nature 1993;361:647–650.
[26] French BA, Mazur W, Geske RS, Bolli R. Direct in vivo gene transfer into porcine myocardium using replication-deficient adenoviral vectors. Circulation 1994;90:2414–2424.
[27] Berkner KL. Expression of heterologous sequences in adenoviral vectors. Curr Top Microbiol Immunol 1992;158:39–66.
[28] Lemarchand P, Jones M, Yamada I, Crystal RG. In vivo gene transfer and expression in normal uninjured blood vessels using replication-deficient recombinant adenovirus vectors. Circ Res 1993;72:1132–1138.
[29] Steg PG, Feldman LJ, Scoazec J-Y, et al. Arterial gene transfer to rabbit endothelial and smooth muscle cells using percutaneous delivery of an adenoviral vector. Circulation 1994;90:1648–1656.
[30] Feldman LJ, Steg PG, Zheng LP, et al. Low-efficiency of percutaneous adenovirus-mediated arterial gene transfer in the atherosclerotic rabbit. J Clin Invest 1995;95:2662–2671.
[31] Smith TA, Mehaffey MG, Kayda DB, et al. Adenovirus mediated expression of therapeutic plasma levels of human factor IX in mice. Nature Genet 1993;5:397–402.
[32] Wang Q, Finer MH. Second-generation adenovirus vectors. Nature Med 1996;2:714–716.
[33] Tripathy SK, Black HB, Goldwasser E, Leiden JM. Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors. Nature Med 1996;2:545–550.
[34] Yang Y, Nunes FA, Berencsi K, Furth EE, Gönczöl E, Wilson JM. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. Proc Natl Acad Sci USA 1994;91:4407–4411.
[35] Yang Y, Ertl HCJ, Wilson JM. MHC class I-restricted cytotxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. Immunity 1994;1:433–442.
[36] Schulick AH, Newman KD, Virmani R, Dichek DA. In vivo gene transfer into injured carotid arteries. Optimization and evaluation of acute toxicity. Circulation 1995;91:2407–2414.
[37] Feldman LJ, Pastore CJ, Aubailly N, et al. Improved efficiency of arterial gene transfer by use of poloxamer 407 as a vehicle for adenoviral vectors. Gene Ther 1997;4:189–198.
[38] Newman KD, Dunn PF, Owens JW, et al. Adenovirus-mediated gene transfer into normal rabbit arteries results in prolonged vascular cell activation, inflammation, and neointimal hyperplasia. J Clin Invest 1995;96:2955–2965.
[39] Ueno H, Li J-J, Tomita H, et al. Quantitative analysis of repeat adenovirus-mediated gene transfer into injured canine femoral arteries. Arterioscler Thromb Vasc Biol 1995;15:2246–2253.
[40] Van Ginkel F, Liu C, Simecka J, et al. Intratracheal gene delivery with adenoviral vector induces elevated systemic IgG and mucosal IgA antibodies to adenovirus and β-galactosidase. Human Gene Ther 1995;6:895–903.
[41] Stratford-Perricaudet LD, Makeh I, Perricaudet M, Briand P. Widespread long-term gene transfer to mouse skeletal muscles and heart. J Clin Invest 1992;90:626–630.
[42] Vilquin J-T, Guérette B, Kinoshita I, et al. FK506 immunosuppression to control the immune reactions triggered by first-generation adenovirus-mediated gene transfer. Human Gene Ther 1995;6:1391–1401.
[43] Engelhardt JF, Ye X, Doranz B, Wilson JM. Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. Proc Natl Acad Sci USA 1994;91:6196–6200.
[44] Yeh P, Dedieu J-F, Orsini C, Vigne E, Denefle P, Perricaudet P. Efficient dual transcomplementation of adenovirus E1 and E4 regions from a 293-derived cell line expressing a minimal E4 functional unit. J Virol 1996;70:559–565.
[45] Qazilbash M, Young N, Liu J. Vectors and target cells for gene therapy of blood diseases. Trends Cardiovasc Med 1996;6:25–30.
[46] Ledley F. Non-viral gene therapy. Curr Opin Biotechnol 1994;5:626–636.
[47] Isner JM, Walsh K, Symes J, et al. Arterial gene therapy for therapeutic angiogenesis in patients with peripheral artery disease. Circulation 1995;91:2687–2691.
[48] Isner JM, Pieczek A, Schainfield R, et al. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. Lancet 1996;348:370–374.
[49] Felgner PL, Ringold GM. Cationic liposome-mediated transfection. Nature 1989;337:387–388.
[50] Takeshita S, Gal D, Leclerc G, et al. Increased gene expression

after liposome-mediated arterial gene transfer associated with intimal smooth muscle cell proliferation. In vitro and in vivo findings in a rabbit model of vascular injury. J Clin Invest 1994;93:652–661.
[51] Lim CS, Chapman GD, Gammon JB, et al. Direct in vivo gene transfer into the coronary and peripheral vasculatures of the intact dog. Circulation 1991;83:2007–2011.
[52] Leclerc G, Gal D, Takeshita S, Nikol S, Weir L, Isner JM. Percutaneous arterial gene transfer in a rabbit model. Efficiency in normal and balloon-dilated atherosclerotic arteries. J Clin Invest 1992;90:936–944.
[53] Zhu N, Liggit D, Liu Y, Debs R. Systemic gene expression after intravenous DNA delivery into adult mice. Science 1993;261:209–211.
[54] Michael SI, Curiel DT. Strategies to achieve targeted gene delivery via the receptor-mediated endocytosis pathway. Gene Ther 1994;1:223–232.
[55] Wilson JM, Grossman M, Wu CH, Chowdhury NR, Wu GY, Chowdhury JR. Hepatocyte-directed gene transfer in vivo leads to transient improvement of hypercholesterolemia in low-density lipoprotein receptor-deficient rabbits. J Biol Chem 1992;267:963–967.
[56] Curiel DT, Agarwal S, Wagner E, Cotten M. Adenovirus enhancement of transferrin-polylysine-mediated gene delivery. Proc Natl Acad Sci USA 1991;88:8850–8854.
[57] Wagner E, Zatloukal K, Cotten M, et al. Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes. Proc Natl Acad Sci USA 1992;89:6099–6103.
[58] Gao L, Wagner E, Cotten M, et al. Direct in vivo gene transfer to airway epithelium employing adenovirus-polylysine-DNA complexes. Human Gene Ther 1994;4:17–24.
[59] Morishita R, Gibbons G, Kaneda Y, Ogihara T, Dzau V. Novel and effective gene transfer technique for study of vascular renin angiotensin system. J Clin Invest 1993;91:2580–2585.
[60] Dzau VJ, Mann MJ, Morishita R, Kaneda Y. Fusigenic viral liposomes for gene therapy in cardiovascular diseases. Proc Natl Acad Sci USA 1996;93:11421–11425.
[61] Morishita R, Gibbons GH, Kaneda Y, Ogihara T, Dzau VJ. Novel in vitro gene transfer method for study of local modulators in vascular smooth muscle cells. Hypertension 1993;21:894–899.
[62] von der Leyen HE, Gibbons GH, Morishita R, et al. Gene therapy inhibiting neointimal vascular lesion: in vivo transfer of endothelial cell nitric oxide synthase gene. Proc Natl Acad Sci USA 1995;92:1137–1141.
[63] Morishita R, Gibbons GH, Ellison KE, et al. Intimal hyperplasia after vascular injury is inhibited by antisense cdk 2 kinase oligonucleotides. J Clin Invest 1994;93:1458–1464.
[64] Morishita R, Gibbons GH, Horiuchi M, et al. A gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth muscle proliferation in vivo. Proc Natl Acad Sci USA 1995;92:5855–5859.
[65] Guzman R, Lemarchand P, Crystal RG, Epstein SE, Finkel T. Efficient and selective adenovirus-mediated gene transfer into vascular neointima. Circulation 1993;88:2838–2848.
[66] Lee SW, Trapnell BC, Rade JJ, Virmani R, Dichek DA. In vivo adenoviral vector-mediated gene transfer into balloon-injured rat carotid arteries. Circ Res 1993;73:797–807.
[67] Riessen R, Isner JM. Prospects for site-specific delivery of pharmacologic and molecular therapies. J Am Coll Cardiol 1994;23:1234–1244.
[68] Lincoff AM, Topol EJ, Ellis SG. Local drug delivery for the prevention of restenosis. Fact, fancy, and future. Circulation 1994;90:2070–2084.
[69] Mitchel JF, Fram DB, Palme II DF, et al. Enhanced Intracoronary thrombolysis with urokinase using a novel, local drug delivery system. In vitro, in vivo, and clinical studies. Circulation 1995;91:785–793.
[70] Camenzind E, Kint P-P, Di Mario C, et al. Intracoronary heparin delivery in humans: Acute feasibility and long-term results. Circulation 1995;92:2463–2472.
[71] Tahlil O, Brami M, Feldman LJ, Branellec D, Steg PG. The dispatch catheter as a delivery tool for arterial gene transfer. Cardiovasc Res 1997;33:181–187.
[72] Fram DB, Aretz T, Azrin MA, et al. Localized intramural drug delivery during balloon angioplasty using hydrogel-coated balloons and pressure-augmented diffusion. J Am Coll Cardiol 1994;23:1570–1577.
[73] Steg PG, Spaulding C, Makowski S, Feldman LJ, Barry JJ. A double blind randomized trial of hydrogel balloon delivery of urokinase during primary angioplasty for acute myocardial infarction. Circulation 1995;92(I):543. abstract.
[74] Wolinsky H, Thung SN. Use of a perforated balloon catheter to deliver concentrated heparin into the wall of the normal canine artery. J Am Coll Cardiol 1990;15:475–481.
[75] Lambert CR, Leone JE, Rowlands SM. Local drug delivery catheters: Functional comparisson of porous and microporous designs. Cor Art Dis 1993;4:469–475.
[76] Hong MK, Wong SC, Farb A, et al. Feasibility and drug delivery efficiency of a new balloon angioplasty catheter capable of performing simultaneous local drug delivery. Cor Art Dis 1993;4:1023–1027.
[77] Fernandez-Ortiz A, Meyer BJ, Mailhac A, et al. A new approach for local intravascular drug delivery: Iontophoretic balloon. Circulation 1994;89:1518–1522.
[78] Fischman D, Leon M, Baim D, et al. A randomized comparison of coronary-stent placement and balloon angioplasty in the treatment of coronary artery disease. N Engl J Med 1994;331:496–501.
[79] Serruys PW, de Jaegere P, Kiemeneij F, et al. A comparison of balloon-expandable-stent implantation with balloon angioplasty in patients with coronary artery disease. N Engl J Med 1994;331:489–495.
[80] Muller DWM, Topol EJ. Implantable devices in the coronary artery: From metal to genes. Trends Cardiovasc Med 1991;1:225–232.
[81] van der Giessen WJ, Serruys PW, Visser WJ. Endothelialization of intravascular stents. J Intervent Cardiol 1988;1:109–120.
[82] Scott NA, Candal FJ, Robinson KA, Ades EW. Seeding of intracoronary stents with immortalized human microvascular cells. Am Heart J 1995;129:860–866.
[83] Dichek DA, Neville RF, Zwiebel JA, Freeman SM, Leon MB, Anderson WF. Seeding of intravascular stents with genetically engineered endothelial cells. Circulation 1989;80:1347–1353.
[84] Flugelman MY, Virmani R, Leon MB, Bowman RL, Dichek DA. Genetically engineered endothelial cells remain adherent and viable after stent deployment and exposure to flow in vitro. Circ Res 1992;70:348–354.
[85] Lambert TL, Dev V, Rechavia E, Forrester JS, Litvack F, Eigler NL. Localized arterial wall drug delivery from a polymer-coated removable metallic stent: Kinetics, distribution, and bioactivity of forskolin. Circulation 1994;90:1003–1011.
[86] Murphy JG, Schwartz RS, Huber KC, Holmes DR. Polymeric stents: Modern alchemy or the future. J Invasive Cardiol 1991;3:144–148.
[87] Dev V, Zeng H, Forrester JS, et al. Microspheres for drug delivery to the arterial wall: A study of kinetics, toxicity, and effects of corticosteroid loaded microspheres. J Am Coll Cardiol 1994;23:19A. abstract.
[88] Slepian MJ, Sawhney A, Pathak CP, et al. In situ photo-polymerized thin hydrogel barriers applied following arterial injury reduce intimal thickening. J Am Coll Cardiol 1994;23:473A. abstract.
[89] Edelman ER, Adams DH, Karnovsky MJ. Effect of controlled adventitial heparin delivery on smooth muscle cell proliferation following endothelial injury. Proc Natl Acad Sci USA 1990;87:3773–3777.

[90] Indolfi C, Avvedimento EV, Rapacciuolo A, et al. Inhibition of cellular ras prevents smooth muscle cell proliferation after vascular injury in vivo. Nature Med 1995;1:541–545.
[91] Simons M, Edelman ER, DeKeyser J-L, Langer R, Rosenberg RD. Antisense c-myb oligonucleotides inhibit intimal arterial smooth muscle cell accumulation in vivo. Nature 1992;359:67–70.
[92] Wolff JA, Malone RW, Williams P, et al. Direct gene transfer into mouse muscle in vivo. Science 1990;247:1465–1468.
[93] Buttrick PM, Kass A, Kitsis RN, Kaplan ML, Leinwand LA. Behavior of genes directly injected into the rat heart in vivo. Circ Res 1992;70:193–198.
[94] von Harsdorf R, Schott R, Shen Y-T, Vatner S, Mahdavi V, Nadal-Ginard B. Gene injection into canine myocardium as a useful model for studying gene expression in the heart of large mammals. Circ Res 1993;72:688–695.
[95] Tsurumi Y, Takeshita S, Chen D, et al. Direct intramuscular gene transfer of naked DNA encoding vascular endothelial growth factor augments collateral development and tissue perfusion. Circulation 1996;94:3281–3290.
[96] Guzman R, Lemarchand P, Crystal R, Epstein S, Finkel T. Efficient gene transfer into myocardium by direct injection of adenovirus vectors. Circ Res 1993;73:1202–1207.
[97] Barr E, Carroll J, Kalynych AM, Tripathy SK, Kozarsky K, Wilson JM. Efficient catheter-mediated gene transfer into the heart using replication-defective adenovirus. Gene Ther 1994;1:51–58.
[98] Giordano FJ, Ping P, McKirnan MD, et al. Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart. Nature Med 1996;2:534–539.
[99] Logeart D, Hatem SN, Inamo J, Haddada H, Perricaudet M, Mercadier JJ. Increased efficiency of gene transfer to cardiac myocytes with recombinant adenovirus by altering the endothelial barrier permeability. Circulation 1996;94(I):590. abstract.
[100] Wilson JM, Birinyi LK, Salomon RN, Libby P, Callow AD, Mulligan RC. Implantation of vascular grafts lined with genetically modified endothelial cells. Science 1989;244:1344–1346.
[101] Chen S-J, Wilson JM, Muller DWM. Adenovirus-mediated gene transfer of soluble vascular cell adhesion molecule to porcine interposition vein grafts. Circulation 1994;89:1922–1928.
[102] Flugelman MY, Jaklitsch MT, Newman KD, Casscells W, Brathauer GL, Dichek DA. Low level in vivo gene transfer into the arterial wall through a perforated balloon catheter. Circulation 1992;85:1110–1117.
[103] Chapman GD, Lim CS, Gammon RS, et al. Gene transfer into coronary arteries of intact animals with a percutaneous balloon catheter. Circ Res 1992;71:27–33.
[104] Riessen R, Rahimizadeh H, Takeshita S, Gal D, Barry JJ, Isner JM. Successful vascular gene transfer using a hydrogel coated balloon angioplasty catheter. Human Gene Ther 1993;4:749–758.
[105] Losordo DW, Pickering JG, Takeshita S, et al. Use of the rabbit ear artery to serially assess foreign protein secretion after site-specific arterial gene transfer in vivo. Circulation 1994;89:785–792.
[106] Nabel EJ, Yang Z, Liptay S, et al. Recombinant platelet-derived growth factor B gene expression in porcine arteries induces intimal hyperplasia in vivo. J Clin Invest 1993;91:1822–1829.
[107] Nabel EG, Shum L, Pompili VJ, et al. Direct gene transfer of transforming growth factor $\beta 1$ into arteries stimulates fibrocellular hyperplasia. Proc Natl Acad Sci USA 1993;90:10759–10763.
[108] Guzman RJ, Hirschowitz EA, Brody SL, Crystal RG, Epstein SE, Finkel T. In vivo suppression of injury-induced vascular smooth muscle cell accumulation using adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene. Proc Natl Acad Sci USA 1994;91:10732–10736.
[109] Chang MW, Barr E, Seltzer J, et al. Cytostatic gene therapy for vascular proliferative disorders with a constitutively active form of the retinoblastoma gene product. Science 1995;267:518–522.
[110] Chang MW, Barr E, Lu MM, Barton K, Leiden JM. Adenovirus-mediated over-expression of the cyclin/cyclin-dependent kinase inhibitor, p21 inhibits vascular smooth muscle cell proliferation and neointima formation in the rat carotid artery model of balloon angioplasty. J Clin Invest 1995;96:2260–2268.
[111] Schulick AH, Dong G, Newman KD, Virmani R, Dichek DA. Endothelium-specific in vivo gene transfer. Circ Res 1995;77:475–485.
[112] Willard JE, Landau C, Glamann DB, et al. Genetic modification of the vessel wall. Comparison of surgical and catheter-based techniques for delivery of recombinant adenovirus. Circulation 1994;89:2190–2197.
[113] Muller DWM, Gordon D, San H, et al. Catheter-mediated pulmonary vascular gene transfer and expression. Circ Res 1994;75:1039–1049.
[114] French BA, Mazur W, Ali NM, et al. Percutaneous transluminal in vivo gene transfer by recombinant adenovirus in normal porcine coronary arteries, atherosclerotic arteries, and two models of coronary restenosis. Circulation 1994;90:2402–2413.
[115] Rome JJ, Shayani V, Flugelman MY, et al. Anatomic barriers influence the distribution of in vivo gene transfer into the arterial wall. Modeling with microscopic tracer particles and verification with a recombinant adenoviral vector. Arterioscler Thromb 1994;14:148–161.
[116] Ohno T, Gordon D, San H, et al. Gene therapy for vascular smooth muscle cell proliferation after arterial injury. Science 1994;265:781–784.
[117] Maillard L, Ziol M, Le Feuvre C, et al. Improved efficiency of percutaneous adenoviral-mediated arterial gene transfer by pretreatment with elastase. J Am Coll Cardiol 1996;27:255A. abstract.
[118] Casscells W, Willerson JT. Amphotropic but not atherotropic: Another caveat for adenoviral gene therapy. J Clin Invest 1995;95:2425–2426.
[119] Perlman H, Maillard L, Krasinski K, Walsh K. Evidence for the rapid onset of apoptosis in medial smooth muscle cells following balloon injury. Circulation, in press.
[120] March K, Madison JE, Trapnell BC. Pharmacokinetics of adenoviral vector-mediated gene delivery to vascular smooth muscle cells: modulation by Poloxamer 407 and implications for cardiovascular gene therapy. Human Gene Ther 1995;6:41–53.
[121] March KL, Gradus-Pizlo I, Wilensky RL, Yei S, Trapnell BC. Cardiovascular gene therapy using adenoviral vectors: distant transduction following local delivery using a porous balloon catheter. J Am Coll Cardiol 1994;23:177A. abstract.
[122] Wilson JM. A pharmacologic rheostat for gene therapy. Nature Med 1996;2:977–978.
[123] Rivera VM, Clackson T, Natesan S, et al. A humanized system fpr pharmacologic control of gene expression. Nature Med 1996;2:1028–1032.
[124] Bohl D, Naffakh N, Heard J-M. Long-term control of erythropoietin secretion by doxyxycline in mice transplanted with engineered primary myoblasts. Nature Med 1997;3:299–305.
[125] Mikawa T, Hyer J, Itoh N, Wei Y. Retroviral vectors to study cardiovascular development. Trends Cardiovasc Med 1996;6:79–86.
[126] Wilson JM. Animal models of human disease for gene therapy. J Clin Invest 1996;97:1138–1141.
[127] Muller DW, Ellis SG, Topol EJ. Experimental models of coronary artery restenosis. J Am Coll Cardiol 1992;19:418–432.
[128] Feldman LJ, Tahlil O, Steg PG. Perspectives of arterial gene therapy for the prevention of restenosis. Cardiovasc Res 1996;32:194–207.
[129] Feldman LJ, Isner JM. Gene therapy for the vulnerable plaque. J Am Coll Cardiol 1995;26:826–835.
[130] Lafont A, Guérot C, Lemarchand P. Prospects for gene therapy in cardiovascular disease. Eur Heart J 1996;17:1312–1317.
[131] Clowes AW, Reidy MA, Clowes MM. Mechanisms of stenosis after arterial injury. Lab Invest 1983;49:208–215.

[132] Bauters C, Meurice T, Hamon M, McFadden E, Lablanche J-M, Bertrand ME. Mechanisms and prevention of restenosis: From experimental models to clinical practice. Cardiovasc Res 1996;31:835–846.

[133] Lafont A, Guzman LA, Whitlow PL, Goormastic M, Cornhill JF, Chisolm GM. Restenosis after experimental angioplasty. Intimal, medial, and adventitial changes associated with constrictive remodeling. Circ Res 1995;76:996–1002.

[134] Mintz GS, Popma JJ, Pichard AD, et al. Arterial remodeling after coronary angioplasty: A serial intravascular ultrasound study. Circulation 1996;94:35–43.

[135] Gibbons GH, Dzau VJ. The emerging concept of vascular remodeling. N Engl J Med 1994;330:1431–1438.

[136] Scott NA, Cipolla GD, Ross CE, et al. Identification of a potential role for the adventitia in vascular lesion formation after balloon overstretch injury of porcine coronary artery. Circulation 1996;93:2178–2187.

[137] Shi Y, Pieniek M, Fard A, O'Brien J, Mannion JD, Zalewski A. Adventitial remodeling after coronary arterial injury. Circulation 1996;93:340–348.

[138] Shi Y, O'Brien JE, Fard A, Mannion JD, Wang D, Zalewski A. Adventitial myofibroblasts contribute to neointimal formation in injured porcine coronary arteries. Circulation 1996;94:1655–1664.

[139] Hoffmann R, Dussaillant GR, et al. Patterns and mechanisms of in-stent restenosis. A serial intravascular ultrasound study. Circulation 1996;94:1247–1254.

[140] Lafont A, Guerot C, Lemarchand P. Which gene for which restenosis?. Lancet 1995;346:1442–1443.

[141] Van Belle E, Maillard L, Couffinhal T, Branellec D, Barry JJ, Feldman LJ. Successful percutaneous adenovirus-mediated gene transfer performed during stent implantation. Circulation 1996;94(1):259. abstract.

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LABORATORY INVESTIGATION

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Direct Myocardial Transfection In Two Animal Models

Evaluation of Parameters Affecting Gene Expression and Percutaneous Gene Delivery

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BACKGROUND: Gene therapy represents a novel approach to the treatment of a variety of disease states. Direct injection of pure untreated DNA into skeletal and cardiac muscle is sufficient to perform gene transfer *in vivo*. Little information is available, however, regarding the extent to which individual parameters of direct gene delivery affect the efficiency of myocardial transfection. Moreover, the fact that all previously reported studies of successful myocardial transfection were performed using open chest surgery to visualize the heart for direct injection of DNA constitutes a potential limitation to clinical applications of myocardial gene transfer. The objectives of the present study were: (a) to determine the extent to which gene expression is altered by varying the amount of DNA and the volume of the delivery vehicle; (b) to study whether protracted expression of DNA persists after direct myocardial transfection; and (c) to test the feasibility of percutaneous, transthoracic myocardial injection of DNA.

EXPERIMENTAL DESIGN: New Zealand white rabbits ($N = 30$) were transfected with the firefly (*Photinus pyralis*) luciferase reporter gene. Twenty-three rabbits were segregated into 5 groups according to the amount of DNA and volume of delivery vehicle injected. These rabbits were sacrificed at 5 days posttransfection to measure the level of luciferase gene expression. Three more rabbits were designated exclusively for *in situ* hybridization. Two rabbits were sacrificed at 5 and 6 months, respectively to evaluate long-term expression. Two additional rabbits were used as controls. Yucatan microswine ($N = 4$) were used to evaluate the feasibility of percutaneous, transthoracic myocardial transfection with β-galactosidase or luciferase reporter genes. Microswine were sacrificed immediately post-sham transfection ($N = 2$), or 5 days postmyocardial transfection ($N = 2$).

RESULTS: Among 11 rabbits, transfection with 10 μg of DNA ($N = 4$), 25 μg of DNA ($N = 3$), and 50 μg of DNA ($N = 4$) in 100 μL of injectate yielded a step-wise but statistically insignificant increase in luciferase activity. Among 16 rabbits, transfection with 50 μg of DNA in injectate volumes of 50 μl ($N = 4$), 100 μl ($N = 4$), 150 μl ($N = 4$), and 300 μl ($N = 4$) yielded a statistically significant ($p < 0.05$) increase in luciferase activity in those hearts transfected with 300 μl compared with those hearts transfected with either 50 or 100 μl. Luciferase activity at 5 and 6 months postmyocardial transfection was 7.14 and 68.8 Turner light units, respectively. *In situ* hybridization confirmed that myocytes represented the site of luciferase expression. Percutaneous myocardial transfection was successfully accomplished with both reporter genes.

CONCLUSIONS: The results of the present study demonstrate that increasing the amount of DNA employed for direct myocardial transfection fails to produce a statistically significant increase in the level of gene expression. In contrast, increasing the volume of injectate used to directly transfect a constant amount of DNA (50 μg) produced significantly augmented expression of the reporter luciferase gene. Luciferase expression was detected 6 months posttransfection. Successful transfection after fluoroscopic-guided direct percutaneous delivery suggests that it may be feasible to deliver genetic material into the myocardium of patients in a similar fashion.

Additional key words: Gene transfer, Myocardium, *In situ* hybridization, Luciferase.

Gene therapy represents a novel approach to the treatment of a variety of disease states. Gene transfer techniques may be used to insert genes into somatic cells, to replace mutated or deleted genes, or to add new genes

with therapeutic potential. Incorporation of genetic material into somatic cells *in vivo* may be achieved directly or indirectly. Indirect gene transfer to cardiovascular tissues has been accomplished by *in vitro* transfection of recombinant DNA to cells growing in tissue culture, followed by introduction of these cells into specific tissue sites of the recipient host *in vivo* (1, 2). Direct gene transfer has been achieved in vascular tissues using DNA combined with a carrier such as cationic liposomes (3), or retroviral vectors (4).

In the case of skeletal muscle, Wolff *et al.* (5) demonstrated that direct injection of pure untreated DNA was sufficient to perform gene transfer *in vivo*. Subsequently, several groups demonstrated that successful *in vivo* gene transfer could also be achieved by direct injection of unescorted DNA into cardiac muscle (6–10). These initial studies involving myocardial transfection were designed to demonstrate the feasibility of the technique (6–10), and to take advantage of this technique to study the expression of DNA constructs under various conditions *in vivo* (7). Surprisingly, two of these seminal reports indicated that the magnitude of expression observed after myocardial transfection exceeded that observed when the same genes were similarly injected into skeletal muscle (7).

The therapeutic potential of this technique was suggested by Acsadi *et al.* (11) who demonstrated expression of human dystrophin in mdx mice (a strain of mice with muscular dystrophy) after intramuscular injection of DNA constructs encoding dystrophin.

Previously reported studies of myocardial transfection, however, have involved limited alternatives of either DNA concentration (1 μg/μl (6), 2 μg/μl (7, 10) or 4 μg/μl (8)) or injection volume (50 μl or 100 μl). Little information is therefore available regarding the extent to which individual parameters of direct gene delivery affect the efficiency of myocardial transfection. Moreover, the fact that all previously reported studies of successful myocardial transfection were performed using open chest surgery to visualize the heart for direct injection of DNA constitutes a potential limitation to clinical applications of myocardial gene transfer.

Accordingly, the purpose of this study was: (a) to determine the extent to which gene expression is altered by varying the amount of DNA and the volume of the delivery vehicle on gene expression; (b) to study whether protracted expression of DNA persists after direct myocardial transfection; and (c) to test the feasibility of percutaneous, minimally invasive myocardial injection of DNA.

EXPERIMENTAL DESIGN

Plasmids

Five different plasmids were employed in the present study. The first involved the *E. coli* β-galactosidase (β-gal) gene (BAG plasmid) under the control of Moloney murine leukemia virus long terminal repeat promoter (12). Two plasmids containing the firefly (*Photinus pyralis*) luciferase gene were used as well. The pRSVLuc plasmid consisted of a full length luciferase cDNA under the control of the Rous sarcoma virus long terminal repeat promoter, subcloned into a vector derived from pBR322 (13). The pRSVLucGEM plasmid consisted of a full length luciferase cDNA under the control of the Rous sarcoma virus long terminal repeat promoter, subcloned into a mammalian expression vector derived from pGEM3 (Promega, Madison, Wisconsin). Two different non-reporter-gene-containing plasmids were used as controls: human acidic fibroblast growth factor subcloned into a pGEM vector (pFGFSN); and rat insulin-like growth factor I receptor subcloned into a pGEM vector (pIGF-ISN).

Myocardial Transfection in Rabbits

Rabbits ($N = 31$) were placed in lateral recumbency, and the heart exposed through a lateral thoracotomy and incision of the pericardium. DNA was injected into the apex of the left ventricle using an insulin syringe with a 28.5-gauge needle (Becton Dickinson, Rutherford, New Jersey). After the apex of the left ventricle was accessed, the needle was advanced along the left ventricular free wall and luciferase plasmid was injected over a period of 5 to 10 seconds. Twenty-three rabbits were divided into 5 groups according to the amount of DNA and volume of delivery vehicle injected. Either pRSVLuc or pRSVLucGEM was arbitrarily assigned for transfection. The amounts of DNA and volume of phosphate-buffered saline (PBS) with 5% sucrose were varied among these 23 rabbits as follows: 10 μg of luciferase plasmid in 100 μl (10 μg/100 μl); 25 μg/100 μl; 50 μg/50 μl; 50 μg/100 μl; 50 μg/150 μl; and 50 μg/300 μl, and two more rabbits, intended to evaluate the feasibility of protracted gene expression, were injected with 100 μg luciferase plasmid in 100 μl PBS/5% sucrose. In addition, 2 rabbits were used as controls. After myocardial injection, the pericardial sac was sutured, a chest tube was placed to drain air and fluids, and the wound was closed. All rabbits were treated with a mixture of 0.1 ml/kg trimethoprim/sulfadiazine (Di-trim 24%, Syntex, West Des Moines, Iowa).

Myocardial Transfection in Microswine

A percutaneous, transthoracic technique was used to inject the hearts of 4 microswine. The first 2 microswine were used to evaluate the feasibility of accurately delivering solution within the ventricular wall by a transthoracic injection. In these 2 microswine, left ventriculography was performed via a catheter that was advanced retrogradely from the femoral artery. A 6-inch long, 18-gauge needle was advanced under fluoroscopic guidance through the skin into the ventricular wall, while the endocardial interface was outlined by the ventriculogram. Correct positioning of the needle within the myocardium of the first microswine was confirmed by injecting a few milliliters of contrast solution and demonstrating fluoroscopically that the dye was retained within the ventricular wall. The same technique was used to inject the myocardium of the second microswine; in this case, however, methylene blue was used instead of contrast solution to mark the site of injection. In two microswine, transfected with the BAG plasmid (one microswine) and with pRSVLuc (one microswine), the left ventricle was not catheterized, and pressure changes recorded from the tip of the needle were used to position the needle within

the ventricular wall. The needle was first advanced into the ventricular cavity, then slowly pulled back until no blood pressure could be recorded through the needle, and no blood could be aspirated; only then was DNA injected through the needle. All microswine were treated with 25 mg/kg of cefazolin (Kefzol, Eli Lilly Indianapolis, Indiana) perioperatively and for 5 days postoperatively.

RETRIEVAL OF TISSUES

All animals were anesthetized, the hearts were exposed as described above, and the animals were killed with intravenous injection of 1 ml/4.5 kg euthanasia solution (Euthanasia-5, Veterinary Laboratories, Lenexa, Kansas). Twenty-five rabbits were sacrificed 5 days after DNA injection. Two other rabbits, used to study long-term expression of transfected DNA, were sacrificed 5 months (one rabbit) and 6 months (one rabbit) after myocardial transfection. Two microswine were sacrificed immediately after sham transfection of the myocardium, the hearts were exteriorized, the site of injection identified, and the tissue containing the injection sites immediately retrieved and fixed in formalin in preparation for histologic examination. The remaining 2 microswine were sacrificed 5 days after DNA injection. All of the hearts were harvested, examined for gross pathologic changes, and rinsed in cold PBS. The rabbit hearts were sectioned as follows (Fig. 3): both atria were trimmed and the free wall of the right ventricle was separated from the left ventricle. The left ventricle (including the interventricular septum) was sectioned along its major axis yielding an apical portion (the site of DNA injection) a mid portion, and a basal portion (farthest from the injection site). The hearts of the last 2 microswine were sliced transversally, perpendicular to the long axis of the heart into slices 1 cm in thickness; each slice was cut open and the resulting strip of myocardium sectioned into 1-cm blocks. Each tissue sample was immediately frozen and stored in liquid nitrogen for further analysis.

IN SITU HYBRIDIZATION

Samples of myocardium were obtained from the apical portion of 6 rabbits transfected with 25 $\mu g/\mu l$ ($N = 1$), 50 $\mu g/100\ \mu l$ ($N = 1$), 50 $\mu g/300\ \mu l$ ($N = 4$), and 100 $\mu g/100\ \mu l$ ($N = 1$) luciferase DNA, as well as from the mid, and basal portions of the left ventricle and from the right ventricle of 3 hearts in which the left ventricle had been transfected with 25 $\mu g/\mu l$, 50 $\mu g/100\ \mu l$, and 50 $\mu g/300\ \mu l$ luciferase DNA. Myocardial samples were also obtained from the apical portion of one rabbit 5 days post-sham transfection (delivery vehicle only (300 μl) without luciferase DNA). The samples were fixed in 4% paraformaldehyde/PBS at 4° C for 2 hours, followed by immersion in 30% sucrose/PBS at 4° C (14). Up to 15 of each 7-μm thick sections from each sample (total 124 tissue sections) were cut onto gelatin coated glass slides and processed for *in situ* hybridization. Radiolabeled sense and anti-sense probes to luciferase DNA were generated from the plasmid pOLuc that contains 1811 luciferase DNA base pairs subcloned into pGEM3 (Promega, Madison, Wisconsin) using SP6 and T7 RNA polimerases, respectively (15). These transcripts were hybridized with 114 of the tissue sections. Sense transcripts were hybridized with 4 tissue sections and used as controls. Autoradiography of the slides was performed by immersing them in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, New York) and exposing them in the dark at 4° C for 1 week. After incubation, the slides were stained with hematoxylin and eosin following which they were examined by light microscopy to identify the presence of silver grains resulting from exposure of the photographic emulsion to the radiolabeled probes.

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM. Statistical differences among the levels of luciferase expression achieved by altering parameters employed for myocardial transfection were compared by analysis of variance. The relationship between luciferase expression and parameters selected for myocardial transfection was also assessed by linear regression analysis. Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

DIRECT MYOCARDIAL TRANSFECTION

Luciferase activity was detected in all hearts transfected with luciferase plasmids. The levels of luciferase activity recorded from the apical portion of the 23 rabbit hearts 5 days post-transfection are summarized in Table 1. These apical sections weighed 1.29 ± 0.06 gm. Fig. 1 illustrates the mean values of luciferase activity among those hearts injected with 10 μg, 25 μg, and 50 μg of luciferase plasmid in a constant volume (100 μl) of fluid carrier. Among 11 rabbits, transfection with 10 μg DNA

TABLE 1. LUCIFERASE ACTIVITY FIVE DAYS POSTMYOCARDIAL TRANSFECTION OF LUCIFERASE DNA IN 23 NEW ZEALAND WHITE RABBITS

Rabbit	Amount of DNA	Volume of injectate	Expression
	μg	*μl*	*TLU*
1	10	100	23,451
2	10	100	8,355
3	10	100	25,925
4	10	100	16,482
5	25	100	10,255
6	25	100	42,339
7	25	100	11,088
8	50	50	21,319
9	50	50	35,218
10	50	50	22,942
11	50	50	7,554
12	50	100	28,885
13	50	100	11,676
14	50	100	40,883
15	50	100	40,120
16	50	150	36,767
17	50	150	67,643
18	50	150	41,397
19	50	150	41,643
20	50	300	170,937
21	50	300	40,367
22	50	300	63,325
23	50	300	77,007

TLU, Turner light units.

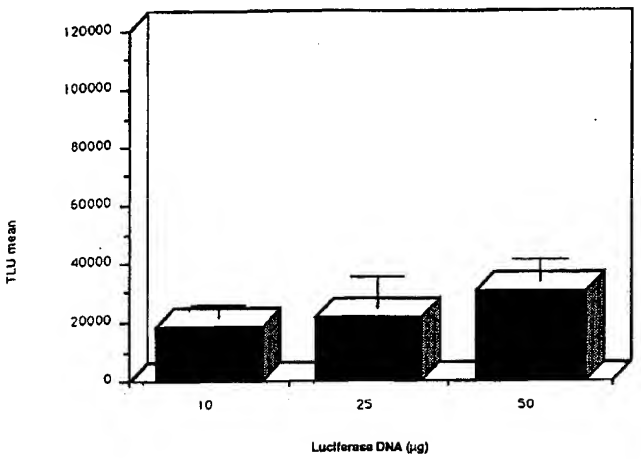

FIG. 1. Luciferase activity expressed in Turner light units (*TLU*), *versus* the amount of DNA injected in a constant volume of injectate (100 µl). Luciferase activity recorded from the apical portion (site of DNA injection) of the left ventricle of rabbit hearts 5 days postmyocardial transfection. Five percent of tissue extracts were assayed, averaged, and multiplied by 20 to calculate the total value of luciferase activity. Differences among the three groups are not statistically significant.

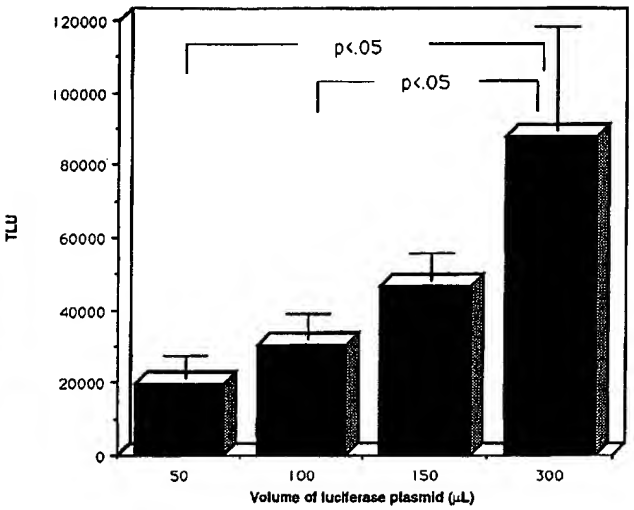

FIG. 2. Luciferase activity, expressed in Turner light units (TLU), *versus* the volume of injectate used to deliver a constant amount of DNA (50 µg). Luciferase activity recorded from the apical portion (site of DNA injection) of the left ventricle of rabbit hearts 5 days postmyocardial transfection. A step-wise increase in luciferase activity is observed, with the level of luciferase activity measured among rabbits injected with 300 µl significantly (analysis of variance, $p < 0.05$) exceeding that observed at 50 µl and 100 µl injectate.

($N = 4$), 25 µg DNA ($N = 3$), and 50 µg DNA ($N = 4$) in 100 µl of injectate yielded 18,553 ± 3,944, 21,227 ± 10,559, and 30,039 ± 6,814 Turner light units (TLU), respectively. While there was a step-wise increase in luciferase activity, the differences observed among these 3 groups were not statistically significant.

Figure 2 shows the mean values of luciferase activity from hearts injected with a constant amount of DNA (50 µg) in increasing volumes of injectate. Among 16 rabbits, transfection with 50 µg DNA in an injectate volume of 50 µl ($N = 4$), 100 µl ($N = 4$), 150 µl ($N = 4$), and 300 µl ($N = 4$) yielded 21,758 ± 5,667, 30,039 ± 6,814, 46,862 ± 7,017, and 87,909 ± 28,960 TLU, respectively. The level of luciferase activity measured among rabbits injected with 300 µl significantly (analysis of variance, $p < 0.05$) exceeded that observed at each 50 and 100 µl injectate. Regression analysis disclosed a linear correlation between the level of luciferase activity and volume of injectate employed ($r = 0.64$, $p < 0.05$). Thus, intramyocardial injection of larger volumes of solution yielded proportionally higher levels of luciferase expression. The coefficient of variation (standard deviation/mean × 100) of luciferase expression for the 23 rabbits ranged between 29.9% and 65.3%.

Myocardial specimens were also retrieved at arbitrary distances from the site of myocardial transfection for the purpose of analyzing luciferase activity as a function of the distance from the site of intramyocardial injection. The highest levels of expression, ranging from 7,554 to 77,007 TLU (mean ± SEM = 38,529 ± 7,198), were detected within the apical portion of the left ventricle, near the site of injection (Fig. 3). The picogram equivalent of light units recorded from the apical segment of the left ventricle ranged between 9.3×10^2 and 4.5×10^3 pg of luciferase. Expression of luciferase activity decreased precipitously beyond the site of injection. Luciferase activity in the mid and basal portions of the left

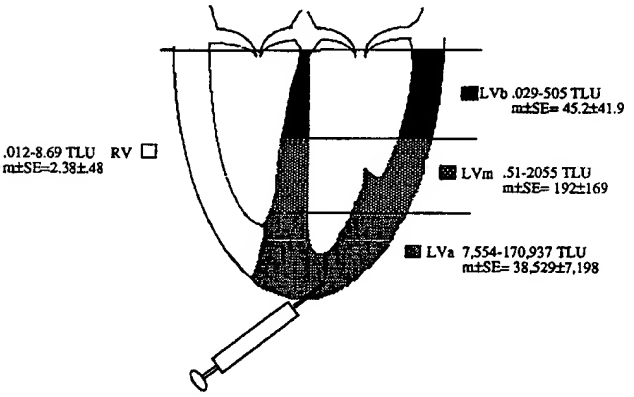

FIG. 3. Distribution of luciferase activity at the apical (*LVa*), mid (*LVm*), and basal (*LVb*) portions of the left ventricle, and within the right ventricle (*RV*) of rabbits, 5 days postmyocardial transfection. The highest levels of luciferase activity, expressed in Turner light units (*TLU*), were detected within the apical portion of the left ventricle, near the site of injection. Expression of luciferase activity decreased precipitously beyond the site of injection.

ventricle and right ventricle was 0.5, 0.11, and 0.02% of values measured in the apical portions of the left ventricle.

Control transfections performed with two unrelated plasmids (pFGFSN and pIGF-ISN) did not express luciferase.

LONG-TERM EXPRESSION OF DNA

To evaluate whether protracted expression of luciferase could be detected, two rabbits injected with 100 µg/100 µl pRSVLuc were sacrificed at 5-month (one rabbit) and 6-month (one rabbit) intervals after myocardial transfection. Retrieval of the heart at necropsy disclosed

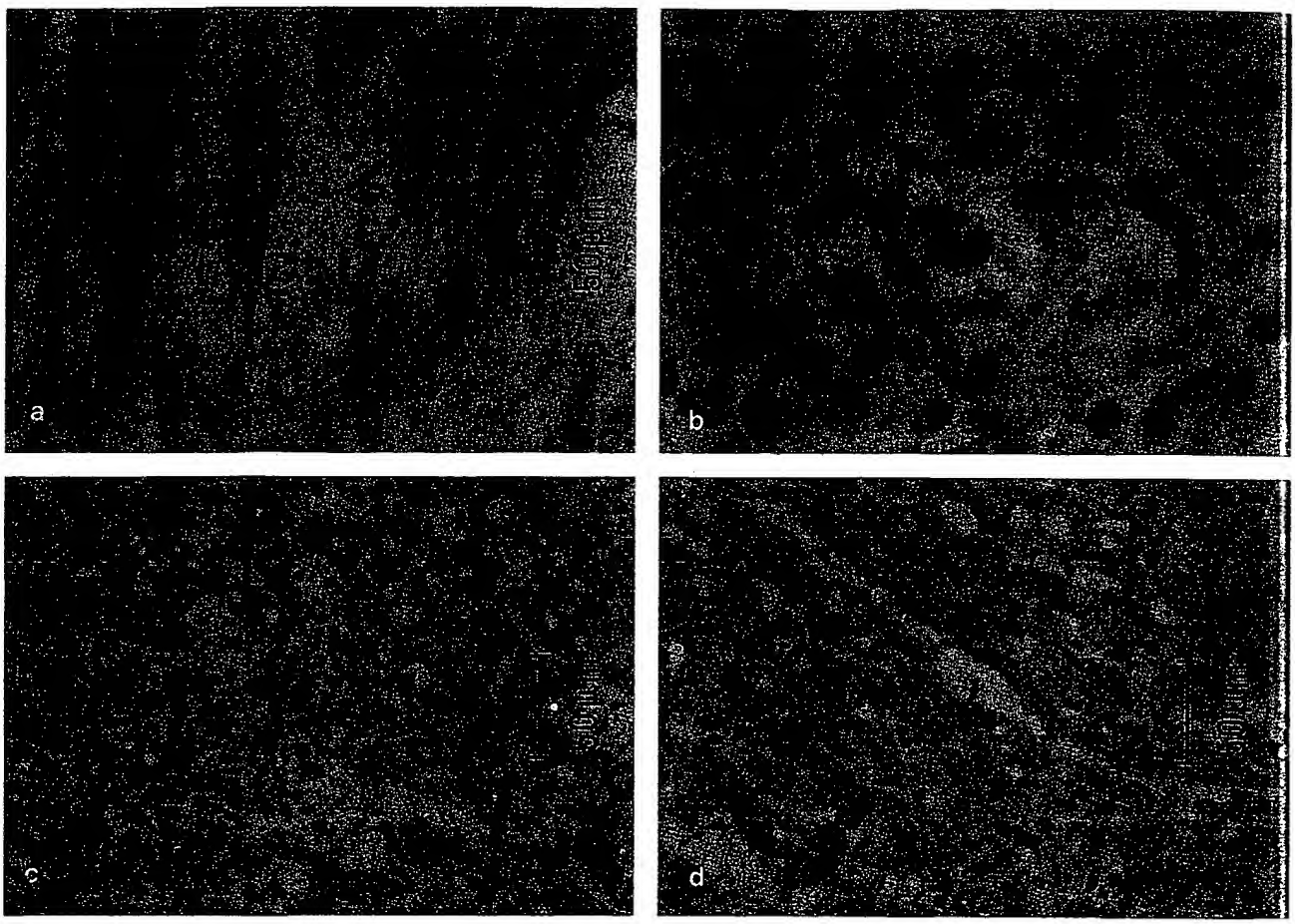

FIG. 4. The results of *in situ* hybridization following transfection of rabbit myocardium. *a*, and *b*, Photomicrographs of frozen sections from rabbit myocardium 5 days postmyocardial transfection with luciferase DNA, incubated with a radiolabeled anti-sense probe generated from pOLuc. Clustering of silver grains (*black dots*), resulting from exposure of the photographic emulsion to the radiolabeled probes, is seen over several myocytes. The clustered silver grains represent positive hybridization of a radiolabeled anti-sense probe with luciferase mRNA. *c*, Photomicrograph of frozen section of the myocardium 5 days postmyocardial transfection with luciferase DNA, incubated with radiolabeled sense probe generated from pOLuc. No hybridization signal similar to that seen in *a* or *b* was detected in myocardial samples incubated with the sense probe. *d*, Likewise no hybridization signal was observed in myocardial sample 5 days post-sham-myocardial transfection (carrier fluid only without luciferase DNA) incubated with the radiolabeled anti-sense probe generated from pOLuc (original magnification ×100). This segment represents a negative control for *in situ* hybridization. Figure 4*a*, ×100; *b*, ×250; *c*, ×100, *d*, ×10.

luciferase activity of 7.14 TLU and 68.8 TLU in these two hearts, respectively.

IN SITU HYBRIDIZATION

In situ hybridization was performed on a total of 124 representative tissue sections from the hearts of 9 rabbits. Fig. 4*a* and *b* illustrate an example of positive hybridization of an anti-sense probe with luciferase mRNA: in this case clustering of silver grains is observed over several myocytes. Incubation of control sections obtained 5 days posttransfection with luciferase DNA, or 5 days post-sham transfection (delivery vehicle only without luciferase DNA) with sense probe (Fig. 4*c*) and with anti-sense probe (Fig. 4*d*), respectively, resulted in a background low uniform hybridization signal.

GROSS PATHOLOGIC EXAMINATION

Gross pathologic examination of 28/31 transfected rabbit hearts was performed 5 days after myocardial injection. Seventeen hearts were normal. In one heart, a focus of myocardial discoloration was observed at the lower third of the left ventricular free wall corresponding to the site of myocardial injection. In 10 hearts, a small (<7 mm^2) focus of myocardial fibrosis was observed at the same site. Retrospective analysis of our data suggested that all but two hearts that had been injected with the CsCl-purified pRSVLucGEM plasmid disclosed no pathologic changes; in contrast, all but one heart in which pathologic changes were observed had been transfected with PEG-purified pRSVLuc.

PERCUTANEOUS MYOCARDIAL INJECTION

Percutaneous injection of the myocardium was successful in 2/2 microswine. Histologic examination of tissue samples obtained from one microswine that was sacrificed immediately after injection of contrast solution showed a relatively large focus of shredded myocytes interdigitating with hemorrhage located immediately be-

FIG. 5. Expression of recombinant β-galactosidase gene in cardiac myocytes 5 days postmyocardial transfection of a Yucatan mycroswine. β-galactosidase activity, represented by dark-blue stain, is seen in tissue section stained with X-gal and with hematoxylin and eosin to demonstrate that β-gal was expressed within the cardiac myocyte. 5-μm section, ×200.

low the epicardial surface. In the second heart injected with methylene blue, pathologic findings were limited to a small focus of subepicardial shredded myocytes.

PERCUTANEOUS MYOCARDIAL TRANSFECTION

One heart injected with the BAG plasmid expressed β-galactosidase (Fig. 5). Gross examination of this heart showed a 1-mm focus of white myocardium corresponding to the site of injection. The other heart in which myocardial transfection was accomplished percutaneously (using the luciferase plasmid) yielded 1,549 TLU. In this latter heart, a 10 × 11 × 18 mm well circumscribed focus of myocardial fibrosis was observed in the left ventricular free wall. Satellite tissue samples were negative for luciferase activity.

Direct injection of naked DNA has been used to transfect the cardiac muscle of rats (6–8), and the skeletal muscle of rats and mice (5, 9, 11). It has been suggested however, that long-term expression may vary among different strains of rats (10), raising the question of whether different species may possess inherently different capacities to express foreign genes after direct injection. The results of the current study supplement data from previous reports by documenting that hearts of not only rabbits, but also swine share the ability to take up and express foreign DNA after direct myocardial transfection.

The results of the present study demonstrated expression of the encoded protein in the hearts of all animals which were transfected, 5 days or more after direct myocardial injection. Increasing amounts of DNA injected in a constant volume of injectate resulted in only a marginal, statistically insignificant increase in luciferase expression. In contrast, levels of luciferase expression consistently increased when increasing volumes of injectate were used to deliver a constant amount of DNA (50 μg). It is likely that increasing volumes of injectate facilitated the spread of DNA within larger areas of the myocardium. Consequently, increasing levels of DNA expression following myocardial transfection with higher volumes of injectate were possibly the outcome of DNA interaction with, and expression of luciferase by, more myocytes. The levels of luciferase expression illustrated in Figure 2 can also be interpreted as a function of DNA concentration. High levels of luciferase expression were obtained at DNA concentrations that were remarkably lower than those reported previously (1 μg/μl (6) or 2 μg/μl (7, 10)). It may therefore be possible to augment DNA expression after direct myocardial transfection by varying the volume of relatively inexpensive, readily available fluid carriers rather than by increasing the amount of DNA.

The variable range of luciferase expression observed among the 23 rabbits sacrificed at 5 days (coefficient of variation = 29.9 to 65.3%) is consistent with results which have been reported after direct gene injection of skeletal muscle (9) and the cardiac muscle (10) of rodents. Wolff *et al.* (9) showed that injection techniques were not responsible for this variability and suggested that variable degradation of the injected DNA might have contributed to variations observed in the expression of DNA.

Luciferase DNA was chosen as the reporter system for most of the present series of experiments for several reasons. The luciferase assay is expeditious, highly specific, extremely sensitive and quantifiable (13). The major disadvantage of this reporter system is that expression of luciferase is not easily localized histochemically, since the tissue sample must be homogenized in order to perform the quantitative assay. Commercially available immunohistochemical assay kits for the detection of luciferase contain fluorescein-conjugated goat anti-rabbit immunoglobulin G antibody. Therefore, the use of this antibody in rabbits is not recommended because of potential nonselective binding of the fluorescein-conjugated antibody. Previous studies have relied upon histochemical localization of β-gal (6, 8) or the finding that genes driven by a myocyte-specific cellular promoter induced gene expression restricted to cardiac myocytes (7). In the present study, we used the β-gal reporter gene and *in situ* hybridization with radiolabeled anti-sense cRNA luciferase transcripts to confirm that myocytes represented the site of luciferase expression.

Detectable levels of expression in the hearts of the two rabbits that were sacrificed at 5- and 6-month intervals after myocardial transfection indicates the feasibility of protracted expression of proteins after myocardial transfection. These findings are in accordance with reports by Leinwand *et al.* (16) who documented the expression of the luciferase reporter gene in rats five to six months after myocardial transfection. Acsadi *et al.* (8) observed that the expression of luciferase plasmid in the cardiac muscle of normal rats was minimally above background 25 days posttransfection, but could be prolonged to 60 days in the cardiac muscle of immunosupressed rats (8). Buttrick *et al.* (11) showed that after cotransfection, chloramphenicol acetyltransferase (CAT) and luciferase reporter genes were both expressed 1 day after direct myocardial injection and the levels of expression peaked between 7 and 10 days. At 17–23 days post-injection, 4/10 hearts expressed the encoded proteins, while 7/10

heart expressed the encoded protein 38 to 60 days after transfection.

Work by Kitsis *et al.* (7) showed that the expression of reporter genes injected into cardiac muscle was 10- to 100-fold higher than the expression of the identical genes after direct injection into skeletal muscles. In the present study the picogram equivalent of light units recorded from the apical segment of the left ventricle (determined by plotting light unit values on a standard curve constructed with a known amount of luciferase) was $\leq 4.5 \times 10^3$ pg. This finding differs by less than one order of magnitude from levels ($\leq$330 pg) that were reported by Wolff *et al.* (5) after injection of 100 μg of luciferase plasmid into the skeletal muscles of rats. Comparison of our findings to those of Wolff *et al.* (5) indicate only modest superiority for myocardial *versus* skeletal muscle transfection efficiency; such comparisons must be, of course, interpreted with caution in view of the fact that the luciferase standard of two laboratories may vary by as much as 100-fold. Nevertheless, the transfection efficiencies that may be achieved in either myocardium or skeletal muscle appear to greatly exceed that may be achieved in vascular smooth muscle (4, 17). The mechanism responsible for such apparently superior results remains enigmatic. It has been suggested that structural factors such as bi- or multinucleated cells, the sarcoplasmatic reticulum, or the rich transverse tubular system of skeletal and cardiac myocytes may facilitate the access of DNA into these cells (5–7). Definitive work regarding this issue, however, remains outstanding.

The histologic findings of damaged myocytes immediately after injection of contrast solution in one microswine may be the result of mechanical disruption caused by a relatively large volume of a highly viscous fluid into the ventricular wall. Injection of PEG-purified DNA was related to a higher incidence of myocyte damage. Although the mechanism responsible for such myocardial alterations is not clear, it is possible that such damage was related to a combination of the mechanical disruption of myocardial tissue caused by the jet of, as well as by the impurities of the injected DNA. It is noteworthy that luciferase activity did not decrease in myocardium characterized by foci of fibrosis near the site of myocardial transfection. Our findings corroborate previous reports suggesting that a relatively small number of myocytes were involved in expression of transfected genes (6, 11) and lead us to speculate that luciferase expression occurs in islands of intact myocytes distributed among foci of myocardial fibrosis.

Successful injection and/or transfection of all hearts following fluoroscopic-guided percutaneous injection suggests that it may be feasible to deliver genetic material into the myocardium of patients in a similar fashion. The accuracy of percutaneous positioning of the needle within the myocardium might ultimately be improved by use of transesophageal echocardiography; alternatively, use of thoracic endoscopy may facilitate accurate and safe delivery of foreign genetic material under direct visual control into epicardial sites. Although not tested in the present investigation, it is possible that administration of genetic material to multiple epicardial sites may increase the yield and/or expression of genetic material.

In conclusion, successful transfection of all hearts after fluoroscopic-guided percutaneous injection suggests that it may be feasible to deliver genetic material into the myocardium of patients in a similar fashion. Expression of injected DNA may be increased by increasing the volume of vehicle in which DNA is delivered rather than saturating the myocardium with high amounts of DNA. Detection of luciferase expression 6 months after transfection suggest that myocardial gene transfer may provide opportunities for protracted gene therapy.

METHODS

PLASMIDS

All plasmids except for pRSVLucGEM were purified in our laboratory by polyethylene glycol (PEG) precipitation (18); pRSVLucGEM was purified twice by CsCl gradient (Lofstrand Laboratories Ltd., Gaithersburg, Maryland). Ethanol precipitation was used to sterilize all plasmids in preparation for myocardial injection, after which, the DNA pellets were reconstituted with sterile phosphate-buffered saline containing 5% sucrose, and stored at 20° C. Before injection, DNA concentration was determined by spectrophotometry.

ANIMALS

Healthy animals from selected and controlled breeding stocks were housed and handled according to protocols approved by St. Elizabeth's Hospital Animal Care and Use Committee, in accordance with the Position of the American Heart Association on Research Animal Use. New Zealand White rabbits ($N = 31$) (Pine Acre Rabbitry Farm, Norwood, Massachusetts) were anesthetized with a mixture of ketamine (10 mg/kg), acepromazine (1.4 mg/kg), and atropine (0.1 mg/kg). The animals were intubated and aseptically prepared for surgery. Anesthesia was maintained with 2% isoflurane (Aerane, Anaquest, Madison, Wisconsin) in oxygen. Heart rate and blood levels of oxygen saturation were continuously monitored transdermally using a Biox pulse oximeter (Model 3740, Ohmeda, Louisville, Colorado). Yucatan microswine ($N = 4$) (Charles River Laboratories, Wilmington, Massachusetts) were anesthetized with a mixture of 20 mg/kg ketamine (Park Davis, Morris Plains, New Jersey), 1.1 mg/kg acepromazine (Avco, Fort Dodge, Iowa) and 0.05 mg/kg atropine (Dexter, Chagrin Falls, Ohio). Anesthesia was maintained with 2% isofurane (Aerane, Anaquest, Madison, Wisconsin) in oxygen.

β-GALACTOSIDASE ASSAY

The area of myocardium which was transfected with β-gal was trimmed and immediately fixed in 1.25% glutaraldehyde and 0.15 M sodium phosphate buffer for 15 minutes and then stained with X-gal (5-bromo-4-chloro-3-indolyl-b-galactopyranoside (Sigma)) for a period of 18 hours as described by others (19). Duplicate sets of 5-μm-thick sections were cut at 12 different levels of the myocardial specimen and processed for light microscopic examination; one set of slide was stained with hematoxylin and eosin.

LUCIFERASE ASSAY

Each tissue sample was weighed, immersed in lysis buffer containing Triton X-100 and dithiothreitol (2:1 volume/weight ratio (Luciferase Assay System, Promega, Madison Wisconsin)), and homogenized (Tissumizer, Tekmar, Cincinnati,

Ohio). The homogenate was spun at 15,000 rpm for 5 minutes in room temperature and tissue extract was removed and its volume was measured. A 20-μl sample of tissue extract was then mixed with 100 μl of a reagent solution containing luciferin and ATP (Luciferase Assay System, Promega). The interaction of luciferase with luciferin in the presence of ATP generates light at 562 nanometers (13). The emission of light, which peaks within seconds, was recorded with a model 20e (Turner Design, Sunnyvale California) luminometer. Five percent of the tissue extracts were assayed, averaged, and multiplied by 20 to calculate the total value of luciferase activity.

Standardization of Luciferase Activity

Standard curves of luciferase activity were generated by plotting light unit values generated by serial dilution of a known amount of luciferase (courtesy of Anna M. Abai, Vical Inc. San Diego, California). Three curves, generated at intervals of several months from multiple samples diluted in phosphate-buffered saline and preserved at −70° C were similar. These dilution curves were used to estimate the amount of luciferase produced following myocardial transfection.

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REFERENCES

1. Nabel EG, Plautz G, Boyce FM, Stanley JC, Nabel GJ. Recombinant gene expression in vivo within endothelial cells of the arterial wall. Science (Washington) 1989;244:1342–4
2. Wilson JM, Birinyi LK, Salomon RN, Libby P, Callow D, Mulligan RC. Implantation of vascular grafts lined with genetically modified endothelial cells. Science (Washington) 1989;244:1344–6
3. Nicolau C, Le Pape A, Soriano, Fargette F, Juhel MF. In vivo expression of rat insulin after intravenous administration of the lyposome-entrapped gene for rat insulin I. Proc Natl Acad Sci USA 1983;80:1068–72
4. Nabel EG, Plautz G, Nabel GJ. Site-specific expression in vivo by direct gene transfer into the arterial wall. Science (Washington) 1990;249:1285–8
5. Wolff JA, Malone RW, Williams PW, Chong W, Acsadi G, Jani A, et al. Direct in vivo transfer into mouse muscle in vivo. Science (Washington) 1990;247:1465–8
6. Lin H, Parmacek MS, Morle G, Bolling S, Leiden JM. Expression of recombinant genes in myocardium in vivo after direct injection of DNA. Circulation 1990;82:2217–21
7. Kitsis R, Buttrick P, McNally E, Kaplan M, Leinwand LA. Hormonal modulation of a gene injected into rat heart in vivo. Proc Natl Acad Sci USA 1991;88:4138–42
8. Acsadi G, Jiao S, Jani A, Duke D, Williams P, Chong W, Wolff JA. Direct gene transfer and expression into rat heart in vivo. New Biol 1991;3:71–81
9. Wolff JA, Williams PW, Acsadi G, Jiao A, Jani A, Chong W. Conditions affecting direct gene transfer into rodent muscle in vivo. BioTechniques 1991;11:474–85
10. Buttrick PM, Kass A, Kitsis RN, Kaplan ML, Leinwand LA. Behavior of genes directly injected into the rat heart in vivo. Circ Res 1992;70:193–8
11. Acsadi G, Dickson G, Love DR, Jani A, Walsh FS, Gurusinghes A, et al. Human dystrophin expression in mdx mice after intramuscular injection of DNA constructs. Science (Washington) 1991;352:815–8
12. Price J, Turner D, Cepko C. Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. Proc Natl Acad Sci 1987;84:156–60
13. DeWet JR, Wood KV, Deluca M, Helsinki DR, Subramani S. Firefly luciferase gene: Structure and expression in mammalian cells. Mol Cell Biol 1987;7:725–37
14. Leclerc G, Isner JM, Kearney M, Simons M, Safian RD, Baim DS, et al. Evidence implicating nonmuscle myosin in restenosis. Use of in situ hybridization to analyze human vascular lesions obtained by directional atherectomy. Circulation 1992;85:543–53
15. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. eds. Current Protocols in Molecular Biology. New York, New York: Greene Publishing Associates and Willey-Interscience, 1989;9.6.12
16. Leinwand LA, Leiden JM. Gene transfer into cardiac myocytes in vivo. Trends Cardiovascular Med 1991;1:271–6
17. Leclerc G, Gal D, Takashita S, Nikol S, Weir L, Isner JM. Percutaneous arterial gene transfer in a rabbit model. Efficiency in a normal and balloon-dilated atherosclerotic arteries. Lab Invest 1992;936–44
18. Sambrook J, Fritsch EF, Maniatis T. Plasmid DNA polyethylene glycol precipitation. In: Nolan C, Ferguson M eds. Molecular Cloning: A Laboratory Manual. 2nd Ed. Cold Spring, New York: Cold Spring Harbor Laboratory Press, 1989;1.40–1
19. Dannenberg AM, Suga M. Histochemical stains for macrophages in cell smears and tissue sections: b-galactosidase, acid phosphatase, non specific esterase, succinic dehydrogenase, and cytochrome oxidase. In: Adams DO, Edelson PJ, Koren MS eds. Methods for studying mononuclear phagocytes. New York: Academic Press, 1981;375–96

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Ischemic/Reperfused Myocardium Can Express Recombinant Protein Following Direct DNA or Retroviral Injection

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H PRENTICE, R. A. KLONER, Y. LI, L. NEWMAN AND L. KEDES. Ischemic/Reperfused Myocardium Can Express Recombinant Protein Following Direct DNA or Retroviral Injection. *Journal of Molecular and Cellular Cardiology* (1996) 28, 133–140. A non-contracting scar following myocardial infarction can adversely affect ventricular topography and hemodynamic function. Gene transfer has the potential to prevent or alter such pathophysiological processes. Normal myocardium is a proven target for delivery of DNA or viral vectors but the potential for gene therapy in ischemic myocardium has not been evaluated. In an initial series of experiments, we determined whether the direct injection of reporter genes into hearts subjected to coronary artery occlusion followed by reperfusion could result in gene expression comparable to the levels observed in non-occluded normal hearts. Anesthetized rats were subjected to 15 min or 60 min of proximal coronary occlusion or sham operation. Luciferase gene under the control of the Rous sarcoma virus promoter was injected directly into the anterior left wall. At 1 week, high expression of luciferase was observed in both the ischemic/reperfused and non-ischemic tissue. Thus DNA transfer by direct injection is possible after ischemic injury and uptake and expression are not impaired. In a second series of experiments, myocardial infarcts in dogs were injected with a β-galactosidase expressing retroviral vector, LNPOZ. Six to 11 days later frozen sections revealed macroscopically visible expression of β-galactosidase activity. Not only can foreign genes be taken up by direct injection of DNA or retroviruses into ischemic/reperfused myocardium but they can be transcribed and the protein synthetic machinery of the injured cells can produce recombinant polypeptides that retain enzymatic activity. These results open the way for the investigation of gene therapy in models of ischemia.

KEY WORDS: Myocardial infarction; Gene therapy; DNA injection; Retrovirus.

Introduction

The potential for exogenously delivered DNA to provide local tissue benefit in diseases of the cardiovascular system, and especially the myocardium, became obvious following an elegant series of experiments that defined striated muscle (Wolff *et al.*, 1990, 1991, 1992; Jiao *et al.*, 1992)—including heart (Lin *et al.*, 1990; Acsadi *et al.*, 1991; Buttrick *et al.*, 1992)—as a tissue with the unique property of being able to take up and express locally injected DNA as recombinant proteins. The cellular distribution of exogenous gene expression following direct myocardial injection is limited to the cardiomyocyte (Buttrick *et al.*, 1992). The direct injection technology can deliver exogenous genes into myocardium that remain under the control of tissue-specific, hormone-inducible or strong viral promoters (Kitsis *et al.*, 1991; Prentice *et al.*, 1994). The proteins encoded by the injected DNA have

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been detected in animal hearts for up to several months (Buttrick *et al.*, 1992).

Direct delivery of genetically engineered DNA to tissues has a wide range of potential therapeutic applications. Indeed, even in its infancy, gene therapy has had highly visible and remarkable promise for providing novel forms of treatment for both hereditary and acquired diseases as pointed out in recent reviews of the topic (Anderson, 1992; Miller, 1992; Mulligan, 1993). Can engineered DNA delivered to a heart that has suffered damage—such as ischemic/reperfusion injury—have potential therapeutic benefit? To answer this question it is first necessary to determine whether such injured tissue can take up exogenous DNA and whether the encoded information can be expressed. Transduction with viral vectors is feasible (Nabel *et al.*, 1989, 1991). Retroviral vectors infect dividing cells preferentially (Miller *et al.*, 1990) and for this reason are not useful to transduce a terminally differentiated cell type such as cardiomyocytes; they would however be expected to transduce proliferating cardiac fibroblasts *in vivo* following ischemic injury.

It has been previously demonstrated (Kloner *et al.*, 1974) that brief (<15 min) coronary occlusion leads to hypoxic cellular responses but not to cell death. Longer periods of occlusion result in a loss of cell viability to varying degrees. Therefore the possibility arises that directly injected DNA may be taken up by cardiomyocytes *in vivo* following limited periods of occlusion followed by reperfusion. In the present experiments, we determined the levels of activity in rat myocardium of a functional reporter enzyme following DNA delivery into hearts subjected to 15 min or 60 min of occlusion and 7 days of reperfusion. We compared randomized ischemic and control groups in an attempt to compensate for intrinsic variations in DNA uptake and delivery. We also performed a feasibility study to establish in principal whether injection of a retroviral vector into a myocardial scar *in vivo* could initiate recombinant gene expression.

Materials and Methods

Rat model of myocardial ischemia and reperfusion

Female Sprague–Dawley rats were anesthetized with 0.1 ml/100 g (body weight) of ketamine (50 mg/ml) and xylazine (10 ng/ml) injected intraperitoneally and the chests were surgically opened to expose the beating heart. The coronary artery was occluded as described (Li and Kloner, 1992) for 15 ($n=12$) of 60 min ($n=6$) followed by reperfusion. DNA injections were performed 15 min later after which the chest was closed. The total amount of DNA delivered to each animal was 50 μg of pRSVLuc and 1% (vol./vol.) Evans blue dye in a volume of 50 μl of normal saline and was delivered at a single site through a 27-gauge needle. The DNA injections were always performed into the anterior free wall of the left ventricle, below the site of the coronary occlusion. This is a site that is always ischemic in our model, assessed by risk zone analysis, histology and electron microscopy (Kloner *et al.*, 1979; Hale *et al.*, 1986; Hale and Kloner, 1987). The septum always remains non-ischemic and it is the free wall that always develops necrosis with prolonged coronary occlusion. Eleven rats served as sham, non-ischemic controls for the 15-min reperfusion group and six rats served as sham, non-ischemic controls for the 60-min group. Animals were sacrificed 7 days after surgery and hearts were removed for analysis of luciferase activity.

Production of myocardial infarction in dogs: a pilot study to determine the feasibility of retroviral transduction in a scar

Mongrel dogs were anesthetized with sodium pentobarbital (35 mg/kg), intubated, and ventilated. Myocardial infarcts were created percutaneously by embolizing alpha helix thrombotic coils (Target Therapeutics) into the left anterior descending coronary artery, under fluoroscopic guidance. Angiography was repeated at approximately 20–40 min to confirm coronary occlusion.

Six to 11 days after coronary occlusion the dogs were anesthetized, intubated, and ventilated. The chest was shaved, prepped with betadine and the animal draped. Using sterile procedure, a thoracotomy was performed in the fifth left intercostal space. The pericardium was incised, exposing the anterior surface of the left ventricle. This allowed visualization of the infarct as pale-yellow, non-contracting tissue in the antero-apical wall of the left ventricle. Retrovirus was injected directly into the wall of the heart. Retroviral supernatants of LNPOZ (see below) were diluted 1:1 with growth medium (DMEM) and injected through a 27-gauge needle into the visible infarct within an area of 1 cm^2. The location of the injections was marked with a suture. Three injections, each of 0.3 ml, were made per infarct. After injection the chest was closed, air was evacuated from the chest, and the dogs allowed to recover for 7–12 days. At 7–12

days, the dogs were killed and hearts excised for analysis. Frozen sections and sections for histology were obtained for immunostaining and histology. All procedures for care of all animals used in these studies were performed in accordance with institutional guidelines. In several animals, the LNPOZ vector was admixed with a second retrovirus rather than growth media. This second retrovirus did not contain a marker gene and is not further evaluated.

Plasmids and choice of DNA injection dose

The plasmid pRSVLuc contains the luciferase gene under the control of the Rous sarcoma virus promoter (Amacher *et al.*, 1993). pRSVLuc (Kitsis *et al.*, 1991) was a kind gift of R. Kitsis and Leinwand (Einstein). In a series of preliminary injections reported earlier (Prentice *et al.*, 1994) in which we compared 10, 25 and 50 μg of pRSVLuc DNA, only the 50 μg dose of DNA gave consistent and significant levels of luciferase expression that could be reproduced. We thus chose to use 50 μg of pRSVLuc DNA for these myocardial injection experiments. Furthermore, 50 μg of pRSVLuc appears to be well below saturating levels of expression and is a low end dose for myocardial DNA injection experiments performed by others. The initial DNA injection experiments from the labs of Leiden (Lin *et al.*, 1990), Wolff (Acsadi *et al.*, 1991) and Leinwand (Kitsis *et al.*, 1991) used 50 to 200 μg of plasmid DNA including the RSV promoter linked to reporter genes. More recent studies used similar doses of DNA (Buttrick *et al.*, 1992; Parmacek *et al.*, 1992).

A study in dogs evaluated a dose response curve using an MSV reporter (von Harsdorf *et al.*, 1993) and found that "there was a linear dose–response relation between the level of gene expression and the quantity of plasmid DNA injected between 10 and 200 micrograms per injection site". A more recent study comparing the expression engendered in porcine myocardium by adenovirus versus pRSVLuc, used 200 μg of DNA at each injection site (French *et al.*, 1994).

Preparation of β-galactosidase encoding amphitrophic retroviruses

β-galactosidase-encoding retrovirus was obtained from a PA317 clone transduced with LNPOZ (gift from A. D. Miller). Amphitrophic retroviruses containing the β-galactosidase coding sequence were obtained as a supernatant from PA317 cells. First, 2 ml of retroviral supernatant derived from infected ψ2 cells was used to transduce PA317 cells at a density of 5×10^5 cells/100-mm dish. Infection was conducted for 3 h after which the culture medium was changed. Twenty-four hours later, cells were split 1:400 for selection in G418 containing medium (1.5 mg/ml) and cultured until antibiotic resistant colonies were visible. One such colony was used as a source of amphotrophic retrovirus. For preparation of retroviral supernatants transduced PA317 cells were grown to a density of 5×10^5 cells/100-mm dish and the cells then cultured in DMEM in the absence of G418 or growth enhancing agents for 24 h after which supernatants were removed and stored at −80°C. The viral titer of LNPOZ was 5×10^5 colony forming units per ml.

Tissue homogenization and luciferase assay

Each heart was excised and the ventricles were washed in ice-cold phosphate-buffered saline. Tissue was homogenized with a Polytron (Kinematic, Switzerland) for 45 s in 1 ml of ice-cold homogenization buffer (Brasier *et al.*, 1989). After centrifugation at 10 000 × g for 10 min, supernatants were frozen at −80°C. For measurements of luciferase activity, samples were assayed for peak light production at 10 s with a Monolight model 2001 luminometer (Analytical Luminescence Laboratory). One hundred microliters of each homogenate (10%) was combined with 350 ml of Buffer B and 100 μl of buffer containing D-luciferin (Brasier *et al.*, 1989) and analysed as described previously (Brasier *et al.*, 1989; Prentice *et al.*, 1994).

β-galactosidase assay

Samples were embedded in OCT compound and 10-μm frozen sections were cut and counterstained with nuclear fast red. Sections of tissue (3 mm) were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature and incubated for 2 h at 37°C in 1 mM 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$.

Histology

For determination of collagen content in infarcted areas tissue was fixed in formalin and embedded in paraffin. Sections were cut and stained with

Table 1 Effects of ischemia/reperfusion on recombinant protein expression

	Non-ischemic		Ischemic	
	Animal*	Light units	Animal*	Light units
15 min	1	28 522	12	3355
	2	4148	13	32 432
	3	628	14	2024
	4	26 361	15	3089
	5	575	16	416
	6	6926	17	3538
	7	7666	18	29 690
	8	936	19	813
	9	154	20	535
			21	236
60 min	Animal**		Animal	
	24	6597	30	246
	25	279	31	7183
	26	7700	32	43 229
	27	4082	33	6080
	28	11 104	34	22 287
			35	524

* Two additional animals in each group had no detectable luciferase activity.
** One additional animal in the non-ischemic group had no detectable luciferase activity.
Luciferase activities (Raw Light Units) for randomized groups of non-ischemic and ischemic animals following 15 or 60 min of occlusion and reperfusion.

hematoxylin and eosin or picrosirius red. Photomicroscopy was performed using Scotchrome 1000 film and a Zeiss microscope.

Results

An *in vivo* rat model of DNA transfer in ischemic/reperfused myocardium

Whereas genes delivered by direct injection into normal hearts enter myocardial cells and are expressed, it has not been determined whether ischemic or otherwise injured myocardium would also be capable of accepting and expressing exogenously delivered DNA. Accordingly, we used a short (15 min) and long (60 min) interval of myocardial ischemia followed by reperfusion in order to engender different degrees of injury to the myocardium which was then followed by direct injection of reporter genes. The short 15 min of occlusion typically results in primarily reversible ischemic injury with minimal or no myocyte necrosis; 60 min of ischemia results in the development of some tissue necrosis. Hearts were excised 7 days after the injection to determine whether the injected gene was expressed. The results are presented in Table 1.

15 min ischemia/reperfusion

We observed RSV-luciferase reporter gene activity after delivery of exogenous DNA to the ischemic area of occluded hearts. The peak light production from these mildly ischemic heart samples did not differ from the levels obtained in non-ischemic control animals. Significant levels of luciferase expression above 100 light units (measured over 10 s) were obtained with 10 of 12 rats in the ischemia/reperfusion group and nine of 11 rats in the non-ischemic control group. Equal amounts of heart tissue homogenate from rats injected with control plasmid [pCAT-Basic vector (Promega)] give background values of 100 light units or less (Prentice *et al.*, 1994). Rats that failed to express are likely to have done so because of faulty injection. Since the amount of DNA injected varied from animal to animal the absolute values of luciferase activity we observed in the injected hearts can be taken only as an indication of whether ischemic myocardium can process and express exogenous genes.

60 min ischemia/reperfusion

One hour occlusion with 7 days of reperfusion also resulted in measurable levels of gene expression. There was a trend (not statistically valid) toward greater expression in this ischemic/reperfusion

Table 2 Summary of infarct status and β-galactosidasestaining for dogs subjected to myocardial infarction

Dog No.	LNPOZ injection	Injection Protocol (interval in days)		Gross infarct at time of injection	β-gal	Comment
		Occlusion-injection	Injection-euthanasia			
2952	+	10	12	+	−	
2953	+	10	12	+	+	
2959	+	6	7	+	+	
3038	+	6	9	−	−	No infarct. No DNA uptake.
3041	+	6	8	+	+	
3043	+	11	7	+	+	

group compared to the non-ischemic group. Five of six rats in the non-ischemic control group and all six rats in the ischemic group expressed significant amounts of luciferase activity.

Injection of retroviruses into the myocardial wall using a dog infarct model

Based on the ability of ischemic myocardium to process injected DNA, we next attempted to determine whether the cells present in a scar forming in the living animal after a myocardial infarction could take up and process a recombinant retrovirus. The density of dividing cells in a myocardial scar is at its peak 5–14 days after infarction and retroviruses preferentially transduce only proliferating cells. Accordingly, we chose this time period to inject β-galactosidase expressing retroviruses into a grossly infarcted segment of myocardium. Six dogs entered the study on retroviral mediated gene transfer. As presented in Table 2, five of six dogs had developed gross myocardial infarctions when observed following thoractomy at 6–11 days post-coronary artery occlusion.

Seven to 12 days after injection of retrovirus into ischemic dog heart the area of gene transfer was examined for Xgal staining. Four out of five dogs with myocardial infarctions that were injected with LNPOZ stained positively for β-galactosidase (Table 2). The dog that was not infarcted was negative for β-galactosidase. This result establishes that retroviral uptake, gene expression and recombinant protein expression is possible in the infarcted area and localizes precisely the injection site.

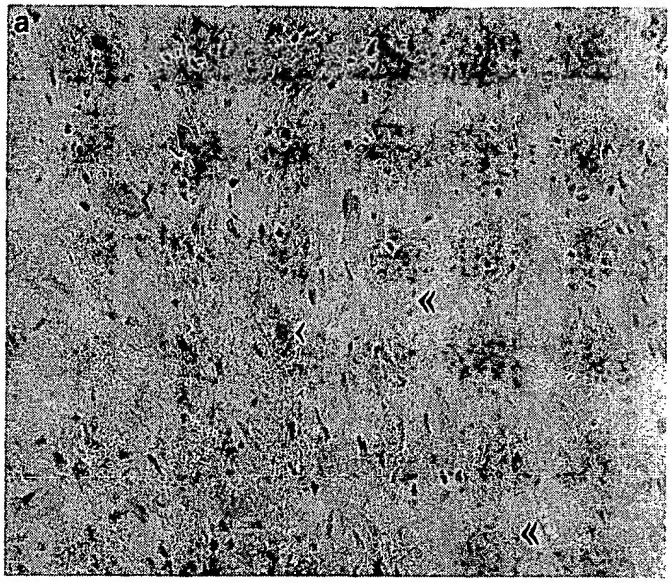
Histology

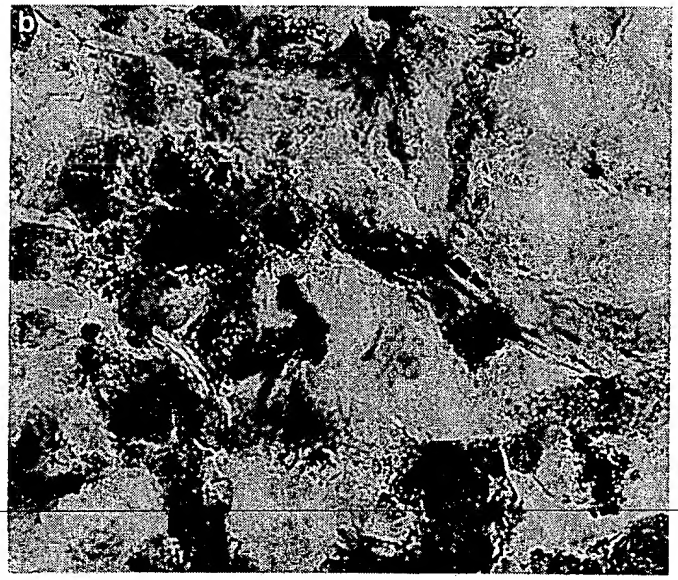
Tissue obtained from the infarcted areas of all dog hearts revealed loss of myocytes with infiltration of fibroblasts and mononuclear cells into the region (Fig. 1a). Picrosirius red staining revealed bright red areas of collagen deposition (Fig. 1c) which were easily distinguished from surrounding viable myocytes at the edge of the infarct which stained yellow. Tissue sections of the infarcted and injected area stained for β-galactosidase activity revealed robust expression of the recombinant gene (Fig. 1b) confined to small regions immediately surrounding the injection sites. The cell type expressing the β-galactosidase activity was uncertain. Uninjected areas and control infarcts showed no trace of β-galactosidase activity.

Discussion

The major conclusions of this study are that the ischemic or infarcted myocardium is a target for gene therapy. Uptake of DNA by rat myocardium following occlusion and reperfusion did not differ from levels obtained with sham-operated rats. This is the first study that we are aware of that shows that ischemic/reperfused myocardium is capable of taking up and expressing foreign genes.

These results open the way for the investigation of direct DNA transfer and gene therapy in models of ischemia. The rat model of left coronary artery occlusion consistently results in ischemia in the anterior left ventricular free wall (Kloner *et al.*, 1979; Hale *et al.*, 1986; Hale and Kloner, 1987). Because it is a model of low coronary collateral flow, this region invariably develops ischemic myocardial necrosis after only 20–30 min of ischemia. It is likely that injections at 15 and 60 min after coronary occlusion resulted in luciferase expression by viable myocytes that were ischemically injured but survived the insult. Additionally, we did not measure the actual number of myocytes that were reprogrammed to express luciferase. The luciferase





activity which was observed could represent a high output of a relatively small number of cells or a lower output of a higher number of cells. Although the length of time that it takes for cells to take up DNA *in vivo* is likely a matter of several hours, during this interval, the myocardial cells that eventually express the gene are injured. That such transiently injured cells can take up and express exogenous DNA has not been previously reported.

The scar that is injected is pleomorphic and contains fibroblasts, endothelial cells, a few macrophages and polymorphonuclear leukocytes as well as a preponderance of mononuclear cells. Some of these cells are capable of proliferating and are the most likely targets for retroviral transduction. Although we have not identified the specific cell type(s) converted in the retroviral mediated gene therapy experiments in which virus was injected into infarct scar 6–11 days after coronary occlusion, proliferating fibroblasts represent one likely target cell. This assumption is based on (a) the near exclusive propensity of retrovial transduction for proliferating cells (Miller *et al.*, 1990), (b) the observation that following ischemic injury in adult myocardium the cardiomyocytes do not proliferate, (c) that at 6–11 days after coronary occlusion the infarct is primarily composed of proliferating cells rather than acellular masses of collagen and (d) that retroviral mediated gene expression fails in non-infarcted myocardium (this work). However, smooth muscle cells, vascular endothelial cell and even infiltrating macrophages cannot be excluded as candidates at this time.

Our experiments indicate not only that foreign genes can be taken up by direct injection into ischemic/reperfused myocardium but, more importantly, that the cellular machinery can transcribe them and can produce recombinant polypeptides that retain enzymatic activity. It remains to be determined whether tissue-specific promoters are also transcribed at high levels and for a similar duration following ischemia and reperfusion. What is novel about these results is the demonstration that retroviral vectors can transduce cells in an organizing scar *in vivo* even when the ground tissue (in this case myocardium) cannot be transduced. These results open the way for the investigation of gene therapy in models of myocardial ischemia, including the delivery of agents that might effect myocardial viability, rate or strength of scar formation or its subsequent vascularization.

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References

Acsadi G, Jiao SS, Jani A, Duke D, Williams P, Chong W, Wolff JA, 1991. Direct gene transfer and expression into rat heart in vivo. *New Biol* 3: 71–81.

Amacher SL, Buskin JN, Hauschka SD, 1993. Multiple regulatory elements contribute differentially to muscle creatine kinase enhancer activity in skeletal and cardiac muscle. *Mol Cell Biol* 13: 2753–2764.

Anderson WF, 1992. Human gene therapy. *Science* 256: 808–13.

Brasier AR, Tate JE, Habener JF, 1989. Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. *BioTech* 7: 1116–1122.

Buttrick PM, Kass A, Kitsis RN, Kaplan ML, Leinwand LA, 1992. Behavior of genes directly injected into the rat heart in vivo. *Circ Res* 70: 193–8.

French BA, Mazur W, Geske RS, Bolli R, 1994. Direct in vivo gene transfer into porcine myocardium using replication-deficient adenoviral vectors. *Circulation* 90: 2414–24.

Hale SL, Kloner RA, 1987. Effect of early coronary artery reperfusion on infarct development in a model of low collateral flow. *Cardiovasc Res* 21: 668–673.

Hale SL, Vivaldi MT, Kloner RA, 1986. Fluorescent microspheres: a new tool for visualization of Ischemic Myocardium in Rats. *Am J Physiol* 251: H863–H868.

Jiao S, Williams P, Berg RK, Hodgeman BA, Liu L, Repetto G, Wolff JA, 1992. Direct gene transfer into nonhuman primate myofibers in vivo. *Human Gene Therapy* 3: 21–33.

Kitsis RN, Buttrick PM, McNally EM, Kaplan ML, Leinwand LA, 1991. Hormonal modulation of a gene

Figure 1 (*opposite*) Photomicrographs of representative histologic and enzymatic assay of infarcted canine myocardium from dog 2959. (a) The infarcted area is largely populated by mononuclear cells that resemble fibroblasts (double arrow heads). There remain scattered foci of residual, red-staining myocytes (single arrow heads). Hematoxylin and eosin staining, ×200. (b) Section of infarct adjacent to injection area, stained for β-galactosidase activity as described in Materials and Methods. The blue stain represents cells with activity. ×600. (c) Picrosirius red staining of the infarcted area reveals dense collagen deposition (red areas) adjacent to residual viable myocardium (yellow). Low power magnification.

injected into rat heart in vivo. *Proc Natl Acad Sci USA* **88**: 4138–42.

KLONER RA, GANOTE CE, WHALEN D, JENNINGS RB, 1974. Effect of a transient period of ischemia on myocardial cells II. Fine structure during the first few minutes of reflow. *Am J Pathol* **74**: 399–422.

KLONER RA, FISHBEIN MC, HARE CS, MAROKO PR, 1979. Early Ischemic Ultrastructural and Histochemical Alterations in the Myocardium of the Rat Following Coronary Artery Occlusion. *Exp Mol Path* **30**: 129–143.

LI Y, KLONER RA, 1992. Cardioprotective effects of ischaemic preconditioning are not mediated by prostanoids. *Cardiovasc Res* **26**: 226–31.

LIN H, PARMACEK MS, MORLE G, BOLLING S, LEIDEN J, 1990. Expression of recombinant genes in myocardium in vivo after direct injection of DNA. *Circulation* **82**: 2217–2221.

MILLER AD, 1992. Human gene therapy comes of age. *Nature* **357**: 455–60.

MILLER DG, ADAM MA, MILLER AD, 1990. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol Cell Biol* **12**: 4239–4242.

MULLIGAN RC, 1993. The basic science of gene therapy. *Science* **260**: 926–932.

NABEL EG, PLAUTZ G, BOYCE FM, STANLEY JC, NABEL GJ, 1989. Recombinant gene expression in vivo within endothelial cells of the arterial wall. *Science* **244**: 1342–4.

NABEL EG, PLAUTZ G, NABEL GJ, 1991. Gene transfer into vascular cells. *J Am Coll Cardiol* **17**: 189B–194B.

PARMACEK MS, VORA AJ, SHEN T, BARR E, JUNG F, LEIDEN JM, 1992. Identification and Characterization of a Cardiac-Specific Transcriptional Regulatory Element in the Slow/Cardiac Troponin C Gene. *Mol Cell Biol* **12**: 1967–1976.

PRENTICE H, KLONER RA, PRYGOZY T, CHRISTENSEN T, NEWMAN L, YUWEI L, KEDES L, 1994. Tissue restricted gene expression assayed by direct DNA injection into cardiac and skeletal muscle. *J Mol Cell Cardiol* **26**: 1393–1401.

VON HARSDORF R, SCHOTT RJ, SHEN T, VATNER SF, MAHDAVI V, NADAL GINARD B, 1993. Gene injection into canine myocardium as a useful model for studying gene expression in the heart of large mammals. *Circ Res* **72**: 688–95.

WOLFF JA, DOWTY ME, JIAO S, REPETTO G, BERG RK, LUDTKE JJ, WILLIAMS P, SLAUTTERBACK DB, 1992. Expression of naked plasmids by cultured myotubes and entry of plasmids into T tubules and caveolae of mammalian skeletal muscle. *J Cell Sci* **103**: 1249–59.

WOLFF JA, MALONE RW, WILLIAMS P, CHONG W, ACSADI G, JANI A, FELGNER PL, 1990. Direct gene transfer into mouse muscle in vivo. *Science* **247**: 1465–8.

WOLFF JA, WILLIAMS P, ACSADI G, JIAO S, JANI A, CHONG W, 1991. Conditions affecting direct gene transfer into rodent muscle in vivo. *Biotechniques* **11**: 474–485.

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Safety and Short-Term Toxicity of a Novel Cationic Lipid Formulation for Human Gene Therapy

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ABSTRACT

Among the potential nonviral vectors for human gene therapy are DNA–liposome complexes. In a recent clinical study, this delivery system has been utilized. In this report, a novel cationic lipid, dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium (DMRIE), has been substituted into the DNA–liposome complex with dioleoyl phosphatidylethanolamine (DOPE), which both improves transfection efficiencies and allows increased doses of DNA to be delivered *in vivo*. The safety and toxicity of this DNA–liposome complex has been evaluated in two species, mice and pigs. The efficacy of DMRIE/DOPE in inducing an antitumor response in mice after transfer of a foreign MHC has been confirmed. No abnormalities were detected after administration of up to 1,000-fold higher concentrations of DNA and lipid than could be tolerated *in vivo* previously. Examination of serum biochemical enzymes, pathologic examination of tissue, and analysis of cardiac function in mice and pigs revealed no toxicities related to this treatment. This improved cationic lipid formulation is well-tolerated *in vivo* and could therefore allow higher dose administration and potentially greater efficiency of gene transfer for gene therapy.

OVERVIEW SUMMARY

Although several viral vectors have been widely applied to the treatment of human disease, the development of nonviral vectors is still in their infancy. In this report, a novel cationic lipid, DMRIE/DOPE, has been incorporated into the DNA–liposome formulation that improves transfection efficiencies and allows up to 1,000-fold higher concentrations of DNA to be administered *in vivo*. In this paper, the safety and toxicity of this formulation is described in two species, mice and pigs, suggesting that it may prove useful for human gene therapy.

INTRODUCTION

ALTHOUGH SEVERAL VIRAL DELIVERY SYSTEMS are available for human gene therapy, nonviral vectors are not as well utilized for gene transfer *in vivo*. We have previously employed a nonviral vector in a human gene therapy protocol in an attempt to stimulate tumor immunity by expression of a foreign histocompatibility antigen in patients with melanoma (G.J. Nabel *et al.*, 1992a,b, 1993). Although several features of such nonviral vectors are attractive for direct gene transfer *in vivo*, a limitation of this system is the relative efficiency of gene transfer. Although the ability to deliver recombinant genes with

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DNA–liposome complexes and to modify biologic effects *in vivo* has been established (E.G. Nabel *et al.*, 1992b, 1993a,b; Plautz *et al.*, 1993), the ability to transduce larger numbers of cells could greatly expand its utility.

There are several potential advantages of DNA–liposome complexes as a nonviral delivery system. Because the DNA is not directly derived from a replication competent virus, its expression is limited. It is unlikely to integrate or propagate in animal cells, thus minimizing concerns related to public health and vector safety. In addition, cationic lipids have been well-tolerated *in vivo* (E.G. Nabel *et al.*, 1992a; Stewart *et al.*, 1992). Based on a variety of animal models (E.G. Nabel *et al.*, 1992a,b, 1993a,b; Stewart *et al.*, 1992) and clinical studies (G.J. Nabel *et al.*, 1992a, 1993) in progress, applications of this technology are likely to expand in the future. Finally, the ability to deliver recombinant genes using catheter-based delivery has further allowed for site-specific gene expression *in vivo*.

Despite the advantages of this technique, some limitations of the present technology remain. Among them has been the relatively low concentration of DNA–liposome complexes that are employed. In previous studies, higher absolute concentrations of DNA–liposome complexes led to aggregation and toxicity following intravenous injection in mice *in vivo* (Stewart *et al.*, 1992). A new formulation (Felgner *et al.*, 1993) is characterized in this report. This DNA–liposome formulation displays a modestly higher transfection efficiency *in vitro*. More importantly, it does not show toxicity at higher absolute concentrations of DNA and lipid. In this report, we characterize the *in vivo* toxicities of this formulation in mice and pigs. We find minimal toxicity at lipid and DNA concentrations up to 1000-fold higher than those used previously. Based on these results, this DNA–liposome formulation may be an appropriate delivery vehicle for gene transfer in humans.

MATERIALS AND METHODS

Plasmids and transfections

A plasmid containing the HLA-B7 gene (G.J. Nabel *et al.*, 1992a, 1993) under the control of the Rous sarcoma virus long terminal repeat (RSV-LTR) was used for transfection of different primary and transformed cell lines and for *in vivo* toxicity analysis. This plasmid was approved for use in our previous human clinical protocol (G.J. Nabel *et al.*, 1992a, 1993). Cells were transfected with dimethylaminoethane-carbamoyl cholesterol (DC-Chol)/dioleoyl phosphatidylethanolamine (DOPE) in an optimal concentration (15 nmol DC-Chol/DOPE; 1 μg DNA in 0.7 ml) as previously described (Gao and Huang, 1991; Stewart *et al.*, 1992). The newer DNA–liposome formulation was prepared by incubation of DNA (5 μg) and dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium (DMRIE)/DOPE (15 nmol) in a final volume of 1 ml in lactated Ringer's solution for *in vitro* transfections.

Animal studies

Adult female mice (strain BALB/c) and domestic pigs were used for all *in vivo* experiments. Experiments in mice were performed to evaluate escalating doses of DNA and liposomes injected intravenously. Intravenous injections were carried out *via* the tail vein using a 21-gauge needle. In mice, for intravenous injections, DNA (0.5–50 μg) and DMRIE/DOPE (1.5–150 nmol) were mixed in a final volume of 0.1 ml immediately prior to injection. Blood was collected from the tail vein before intravenous injection of the DNA–liposomes and 10 days later. At about 2 weeks following injection, liver, kidney, lung, heart, and brain were removed for histologic analysis and extracted for polymerase chain reaction (PCR) DNA amplification using primers and conditions described previously (G.J. Nabel *et al.*, 1993) in one group of mice ($n = 5$). In a second group, injections were repeated at 2-week intervals for a total of three times. Blood was collected before the first injections and 6 weeks later, at which time organs were removed and analyzed by histology and PCR.

Additional toxicity studies were performed in 12 pigs to evaluate the local and systemic effects of three doses of DNA and liposomes when delivered by a catheter to a local peripheral artery segment. Intra-arterial gene transfer was performed using a double balloon catheter (USCI, Bard, Inc., Billerica, MA) with methods previously described (Nabel *et al.*, 1990). In these experiments, the plasmid containing the HLA-B7 gene was mixed with DMRIE/DOPE in three concentrations: HLA-B7 DNA 0.5 μg and DMRIE/DOPE 1.5 nmol (2 pigs); HLA-B7 DNA 5 μg and DMRIE/DOPE 15 nmol (4 pigs); and HLA-B7 DNA 50 μg and DMRIE/DOPE 150 nmol (6 pigs). The animals were sacrificed at 17–21 days. The following evaluations were performed. Tissue analysis for PCR and histology were done 17–21 days following gene transfer to determine the effectiveness of gene transfer and the effects of gene expression on tissues. Serum chemistries were measured before gene transfer and 17 days later to examine effects of DNA and DMRIE/DOPE liposomes on liver, renal, and cardiac function.

The PCR reactions were performed as previously described (G.J. Nabel *et al.*, 1993) with a 2-min annealing and extension at 72°C and 1 min of dissociation of 94°C. Plasmid DNA was used as positive control (1 ng). The sensitivity of detection has been estimated to be between 1 copy per 10^3–10^5 genomes (Stewart *et al.*, 1929). Serum samples were stored frozen at −20°C for measurement of tissue-specific enzymes and routine biochemical parameters.

Studies of cardiac toxicity

The effects of intravenous DNA–liposome complexes on cardiac tissue were assessed by measurement of total creatine phosphokinase (CPK), CPK isoenzymes, and electrocardiography (EKG) on 15 mice prior to and following DNA–liposome injection. Total CPK values were determined prior to injection. Mice received HLA-B7 DNA–liposome conjugates prepared as described above (0.1 cc) injected into the tail vein. CPK measurements were made on serum samples obtained 16 hr after injection. Control mice were injected with 0.1 cc of saline, and total CPK was measured at 16 hr.

As an independent parameter to evaluate cardiac function, EKG measurements were performed prior to DNA–liposome complex or saline injection, during injection, and 1 and 5 min following injection. The mice were anesthetized and surface lead electrodes were attached to the four limbs using alcohol.

Surface lead I or II was monitored at a chart speed of 50 mm/sec. After the baseline EKG was obtained, continuous measurements were made during injection.

Direct arterial gene transfer in vivo

Direct arterial transfection was performed in 12 pigs using methods as previously described in peripheral iliofemoral arteries using a catheter (E.G. Nabel *et al.*, 1990, 1992b). In this study, the expression of the human recombinant gene was analyzed up to 17 days following direct gene transfer in pigs. Genomic DNA was prepared from transfected and nontransfected arteries, ovary or testes, heart, lung, liver, spleen, kidney, and skeletal muscle by standard proteinase K digestion and phenol and chloroform extraction conditions. Oligonucleotide primers were synthesized to generate the 525-bp fragment as described above.

Analysis of organ toxicity

Organ specimens, including transfected and nontransfected artery, ovary or testes, brain, heart, lung, liver, spleen, kidney, and muscle were obtained when the animals were sacrificed, fixed in formalin, embedded in paraffin, and stained in hematoxylin and eosin. Representative sections were examined by an experienced pathologist (D.G.) in a blinded fashion.

Serum from mice or pigs was obtained prior to sacrifice, frozen at −20°C, and biochemical analyses were performed (Roche Biomedical Laboratories, Dublin, OH). Mean values and standard errors of the mean (SEM) prior to and following gene transfer were compared using a two-tailed paired *t*-test. Results were considered statistically significant if $p \leq 0.05$.

RESULTS

Comparison of transfection efficiencies of DMRIE/DOPE and DC-Chol/DOPE in vitro

To compare the efficacy of DMRIE/DOPE to the DC-Chol/DOPE, a variety of cell lines were transfected *in vitro*. Among the cell lines, the DMRIE/DOPE formulation showed an improved transfection efficiency ranging from two- to seven-fold, depending on the lines that were examined (Fig. 1). In general, the relative improvement with DMRIE/DOPE was most effective in cell lines that were difficult to transfect with both cationic lipids, *e.g.*, MCA 205 fibrosarcoma. It is also important to note that a higher concentration of plasmid DNA was used with DMRIE/DOPE (5 μg) than DC-Chol/DOPE (1 μg) because this higher concentration of plasmid was toxic to cells *in vitro* with DC-Chol/DOPE (Stewart *et al.*, 1992). Thus, DMRIE/DOPE is not necessarily more potent than DC-Chol/DOPE, but its relative lack of toxicity allows larger quantities of DNA to be used *in vitro* and *in vivo*.

In Fig. 2, an example is provided regarding the potential for improved therapy with this new liposome formulation. A subclone of MCA 205, a murine fibrosarcoma (H-2K^b), was poorly transfected by DC-Chol/DOPE liposome. Using the DMRIE/

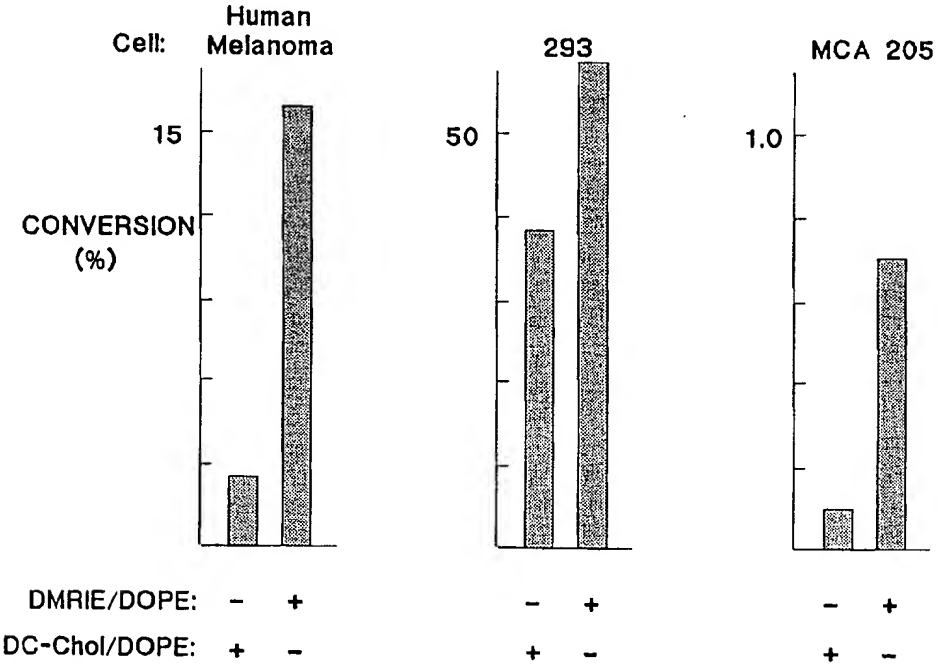

FIG. 1. Improved transfection efficiency of DMRIE/DOPE DNA–liposome complex. Cells were incubated with DNA–liposomes reconstituted in Ringer's lactate solution for 1.5 hr and analyzed after 36 hr. Transfection efficiencies of DMRIE/DOPE and DC-Chol/DOPE are shown. Percent transfected cells were analyzed by X-gal staining (Nabel *et al.*, 1989). Standard deviations were <10%.

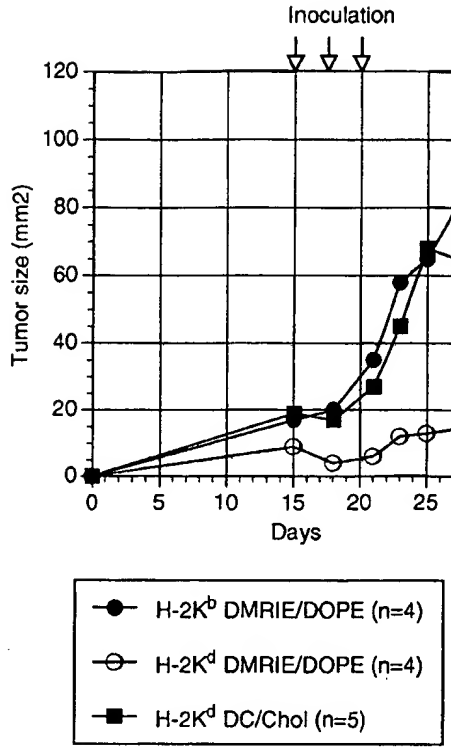

FIG. 2. Improved antitumor effect with DMRIE/DOPE liposomes compared to DC-Chol/DOPE. C57/BL6 mice (H-2K^b) were inoculated with MCA 205 fibrosarcoma tumor cells subcutaneously in the left posterior hind flank on day 0. Presensitization was performed by subcutaneous injection of BALB/c (H-2K^d) spleen cells, 5×10^6 on day -6, and 2×10^6 on day $+1$. Tumors were injected (0.1 ml total volume) on days 15, 18, and 20 with: (i) CMV H-2K^b 5 μg and DMRIE/DOPE 15 nmol (-●-); (ii) CMV H-2K^d 5 μg and DMRIE/DOPE 15 nmol (-○-); and (iii) CMV H-2K^d 5 μg and DC/Chol/DOPE 15 nmol (-■-). Tumor size, calculated as the product of two perpendicular diameters, was measured at the indicated times. Standard deviations for each point were <20%.

DOPE formulation, a marked antitumor effect was obtained after introduction of a foreign MHC gene (H-2K^d) that was not seen with DC-Chol (Fig. 2).

Distribution of DNA–liposome complexes after intravenous and intra-arterial administration

To characterize the distribution of DNA–liposome complexes after injection *in vivo*, the complexes were prepared and injected into the tail vein of BALB/c mice. The RSV HLA-B7 plasmid used for the human gene therapy protocol was utilized for these studies. Increasing concentrations of DNA–liposome complexes were tested in three groups. The lowest dose represented a 10-fold higher initial dose than for the human gene therapy trial (0.5 μg plasmid DNA; 1.5 nmol DMRIE/DOPE) based on a weight/body surface area ratio. The highest dose was 1,000-fold greater. PCR analysis of DNA from major murine organs, including heart, lung, brain, liver, and kidney, showed that the injected DNA was detected in multiple organs, including the lung and heart, with these mice after 10 days (Table 1A). However, the presence of DNA in these organs was not associated with organ pathology (see below). The localization of DNA–liposome complexes in different tissues has been described previously (Stewart *et al.*, 1992), where it was found transiently in cells of the reticuloendothelial cell system.

Localized gene transfer to arterial segments was performed in 12 pigs at three doses, HLA-B7 DNA 0.5 μg and DMRIE/DOPE 1.5 nmol (2 pigs), HLA-B7 DNA 5.0 μg and DMRIE/DOPE 15 nmol (4 pigs), and HLA-B7 DNA 50 μg and DMRIE/DOPE 150 nmol (6 pigs). The right and left iliofemoral arteries were transfected in the 12 pigs, for a total of 24 arteries. PCR analysis from these pigs demonstrated HLA-B7 DNA in 20 of 24 arteries, 3 of 4 arteries transfected at the 0.5 μg/1.5 nmol dose, 6 of 8 arteries transfected at the 5.0 μg/15 nmol dose, and 11 of 12 arteries transfected at the 50 μg/150 nmol dose. In addition, PCR analysis did not demonstrate HLA-B7 DNA in major organs 17 days following intraarterial gene transfer (Table 1B).

TABLE 1. LOCALIZATION OF RECOMBINANT GENES BY PCR AFTER INTRODUCTION *In Vivo* IN MICE AND PIGS

A. DNA/liposome	*Brain*	*Heart*	*Lung*	*Liver*	*Kidney*	*Spleen*	*Muscle*	*Ovary*
0.5 μg/1.5 nmol	2/6	5/6	5/6	2/6	3/6	2/6	0/6	0/6
5.0 μg/15 nmol	2/6	1/6	2/6	3/6	2/6	4/6	0/6	0/6
50 μg/150 nmol	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

Mice were injected with 0.1 ml of the indicated concentrations of HLA-B7 plasmid and DMRIE/DOPE, and DNA was extracted from the indicated tissues at 10 days for analysis by PCR. Mice received injections by tail vein. The number of animals which tested positive relative to the total number analyzed is indicated.

B. DNA/liposome	*Heart*	*Lung*	*Liver*	*Kidney*	*Spleen*	*Muscle*	*Nontransfected artery*	*Ovary*
0.5 μg/1.5 nmol	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
5.0 μg/15 nmol	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
50 μg/150 nmol	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

Pigs were treated with indicated concentrations of plasmid DNA and DMRIE/DOPE by arterial gene transfer (Nabel *et al.*, 1993a,b). DNA was extracted from the indicated tissues at 17 days and analyzed as in A.

TABLE 2. EVALUATION OF SELECTED SERUM ENZYMES AND CHEMISTRIES BEFORE AND AFTER A SINGLE INTRAVENOUS INJECTION OF HLA-B7 DNA–LIPOSOMES IN MICE

Plasmid (μg): / *DMRIE/DOPE (nmol):*	*0.5* / *1.5*		*5* / *15*		*50* / *150*	
	Pre	*Post*	*Pre*	*Post*	*Pre*	*Post*
Albumin (g/dl)	3.4 ± 0.4	3.3 ± 0.3	3.3 ± 0.4	3.2 ± 0.3	3.1 ± 0.1	3.3 ± 0.2
Alk Phos (IU/liter)	146 ± 21	152 ± 13	164 ± 14	170 ± 17	172 ± 8	193 ± 23
Amylase (U/liter)	2,261 ± 31	2,337 ± 192	2,395 ± 277	2,465 ± 339	2,612 ± 166	2,226 ± 217
Bilirubin (mg/dl)	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0.1	0.1 ± 0
BUN (mg/dl)	20 ± 4	25 ± 2	35 ± 6	26 ± 3	24 ± 9	20 ± 0
Creatinine (mg/dl)	0.5 ± 0.2	0.6 ± 0.3	0.5 ± 0.2	0.3 ± 0	1.0 ± 0.2	0.2 ± 0
SGOT (IU/liter)	71 ± 8	60 ± 7	57 ± 3	61 ± 6	40 ± 12	60 ± 8
Total protein (g/dl)	6.3 ± 0.8	5.2 ± 0.2	5.4 ± 0.8	5.4 ± 0.6	5.0 ± 0	4.5 ± 0.2

Blood samples were obtained from BALB/c female mice ($n = 5$) prior to intravenous injection (Pre) and 10 days after (Post) tail vein injection with the indicated concentrations of DNA–liposome complexes. Serum enzyme and chemistry values were analyzed (Roche Biomedical Laboratory), and mean values and standard deviations are shown.

Organ toxicity

To determine whether the introduction of DNA–liposome complexes *in vivo* produced toxicity to major organ systems, several serum biochemical parameters were evaluated either after a single injection or after three separate injections made at 2-week intervals in mice and pigs. The previous studies regarding the long-term toxicity and immunologic consequences of expression of a foreign MHC gene have been previously reported with no adverse effects noted (E.G. Nabel *et al.*, 1992a). Analysis of serum enzymes and protein from liver, kidney, bone, and pancreas revealed no significant changes 10 days after a single injection (Table 2) or 14 days after three treatments administered at 2-week intervals (Table 3). Similar analyses were performed in pigs that received arterial gene transfer with DNA–liposome complexes, and no biochemical abnormalities were observed (Table 4). Pathology analysis of tissues from these animals showed occasional incidental changes unrelated to the introduction of DNA–liposome complexes (Table 5). The incidental findings, including occasional peribronchial lymphoid aggregates in the lung or liver were seen in control animals that did not receive DNA–liposome treatments in previous studies (E.G. Nabel *et al.*, 1992a; Stewart *et al.*, 1992). Similarly, no pathological abnormalities were detected after three treatments with DNA–liposome complexes in mice (data not shown), analogous to the protocol used in our human clinical trial (G.J. Nabel *et al.*, 1992a, 1993). In addition, toxicity studies were performed in pigs following direct arterial gene transfer. These pathology studies demonstrated that the administration of DNA–liposome complexes intra-arterially was well-tolerated *in vivo*, with no adverse responses detected biochemically (Table 4) or in tissues (Table 5).

Cardiac toxicity

As in previous studies, plasmid DNA was detected after intravenous injection in the heart by PCR analysis. Therefore, we examined whether this formulation caused significant acute or chronic toxicity from this treatment. We first evaluated the

TABLE 3. EVALUATION OF SELECTED SERUM ENZYMES AND CHEMISTRIES BEFORE AND AFTER THREE INTRAVENOUS INJECTIONS OF HLA-B7 DNA–LIPOSOME COMPLEXES IN MICE

Plasmid (μg): / *DMRIE/DOPE (nmol):*	*0.5* / *1.5*		*5* / *15*		*50* / *150*	
	Pre	*Post*	*Pre*	*Post*	*Pre*	*Post*
Albumin (g/dl)	3.4 ± 0	2.9 ± 0	3.1 ± 0.2	2.9 ± 0	3.2 ± 0.1	3.2 ± 0.2
Alk Phos (IU/liter)	152 ± 8	113 ± 10	161 ± 16	124 ± 14	170 ± 8	182 ± 11
Amylase (U/liter)	2,246 ± 17	2,319 ± 185	2,213 ± 172	2,223 ± 116	2,585 ± 178	2,183 ± 277
Bilirubin (mg/dl)	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.3 ± 0
BUN (mg/dl)	21 ± 3	18 ± 3	37 ± 7	23 ± 8	26 ± 10	22 ± 3
Creatinine (mg/dl)	0.7 ± 0	0.4 ± 0.3	0.4 ± 0.3	0.4 ± 0.3	1 ± 0	0.3 ± 0
Phosphorous (mg/dl)	6.7 ± 0.5	6.2 ± 0.5	7.2 ± 0.5	5 ± 0.8	7.6 ± 0.9	7.7 ± 1.8
SGOT (IU/liter)	69 ± 10	53 ± 15	56 ± 3	69 ± 28	41 ± 13	46 ± 2
SGPT (IU/liter)	35 ± 3	27 ± 9	31 ± 8	37 ± 5	25 ± 4	26 ± 2
Total protein (g/dl)	6.3 ± 1	4.8 ± 0.3	5.4 ± 0.9	5.1 ± 0.5	5.0 ± 0	4.8 ± 0

Blood samples were obtained from BALB/c female mice ($n = 5$) prior to intravenous injection (Pre) and 14 days after (Post) the third injection of DNA–liposome complexes at the indicated concentrations. Serum enzyme and chemistry values were analyzed (Roche Biomedical Laboratory), and mean values and standard deviations are shown.

TABLE 4. EVALUATION OF SELECTED SERUM ENZYMES AND CHEMISTRIES BEFORE AND AFTER ARTERIAL GENE TRANSFER OF HLA-B7 DNA–LIPOSOME COMPLEXES IN PIGS

Plasmid (μg):	*5*		*50*	
DMRIE/DOPE (nmol):	*15*		*150*	
	Pre	*Post*	*Pre*	*Post*
Albumin (g/dl)	3.2 ± 0.4	3.2 ± 0.2	3.3 ± 0.3	3.4 ± 0.3
Alk Phos (IU/liter)	298 ± 62	186 ± 29	241 ± 97	206 ± 51
Amylase (U/liter)	1,799 ± 139	2,170 ± 470	2,269 ± 605	2,527 ± 1,297
Bilirubin (mg/dl)	0.14 ± 0.05	0.22 ± 0.04	0.10 ± 0	0.14 ± 0.05
BUN (mg/dl)	8 ± 3	9 ± 3	6 ± 1	10 ± 2
Calcium (mg/dl)	9.3 ± 0.2	9.3 ± 0.4	9.5 ± 0.6	9.5 ± 1.0
Chloride (mEq/liter)	101 ± 1	99 ± 5	103 ± 1	101 ± 5
Creatinine (mg/dl)	0.9 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	1.2 ± 0.2
Glucose (mg/dl)	111 ± 57	134 ± 54	102 ± 56	116 ± 22
LDH (IU/liter)	538 ± 146	507 ± 161	425 ± 62	489 ± 173
Phosphorous (mg/dl)	10.3 ± 0.7	7.9 ± 0.8	10.5 ± 1.4	10.9 ± 4.1
Potassium (mEq/liter)	5.1 ± 1.0	4.4 ± 0.8	4.5 ± 0.4	4.8 ± 1.1
SGOT (IU/liter)	28 ± 8	44 ± 30	27 ± 13	43 ± 30
SGPT (IU/liter)	29 ± 14	42 ± 12	36 ± 9	40 ± 7
Sodium (mEq/liter)	139 ± 2	135 ± 5	143 ± 2	142 ± 9
Total protein (g/dl)	5.0 ± 0.1	5.4 ± 0.5	5.7 ± 0.5	5.7 ± 0.5

Blood samples were obtained from pigs (n = 10) prior to (Pre) and 17 days after (Post) arterial gene transfer of DNA–liposome complexes at the indicated concentrations (n = 5 for each concentration). Serum enzyme and chemistry values were analyzed (Roche Biomedical Laboratory), and mean values and standard errors of the mean are shown. All values fall within the range of normal limits (Nabel *et al.*, 1992a).

potential for acute effects of this treatment by examining the electrocardiogram before, during, and after injection on DMRIE/DOPE plasmid complexes. As observed with DC-Chol liposomes, this analysis revealed no abnormalities in rate or rhythm after intravenous injection (Fig. 3). As a further evaluation of cardiac toxicity, CPK measurements were performed in mice before or after injection with DNA–liposome complexes. No significant changes in CPK levels were noted pre- or post-injection (Table 6). In addition to these analyses, histopathological analysis of tissue was performed. No significant pathological abnormalities were detected at 14 days to 6 weeks after injection (Table 5). In summary, despite the detection of complexes by PCR within the myocardium, there was no acute or chronic cardiac toxicity from this treatment.

DISCUSSION

The potential of nonviral vectors as a delivery vehicle for human gene therapy has expanded over the past several years.

TABLE 5. HISTOLOGICAL ANALYSIS OF TISSUE FOLLOWING INTRODUCTION OF DNA–LIPOSOMES *In Vivo*

Species:	*Mice*			*Pigs*	
μg DNA/nmol liposome:	*0.5/1.5* (n = 5)	*5/15* (n = 5)	*50/150* (n = 14)	*5/15* (n = 4)	*50/150* (n = 6)
Heart	Normal	Normal	Normal	Normal	Normal
Lung					
[a]Focal peribronchial lymphoid aggregates	2/5	Normal	3/14	3/4	3/6
Liver					
[a]Focal portal mononuclear inflammatory aggregates	2/5	2/5	4/14	Normal	3/6
Kidney					
[a]Focal interstitial mononuclear inflammatory cells	Normal	Normal	1/14	Normal	Normal
Spleen	Normal	Normal	Normal	Normal	Normal
Skeletal muscle	Normal	Normal	Normal	Normal	Normal
Nontransfected artery	ND	ND	ND	Normal	Normal
Ovary	Normal	Normal	Normal	Normal	Normal

ND, not determined.
[a]Minor pathologic changes of no clinical significance.

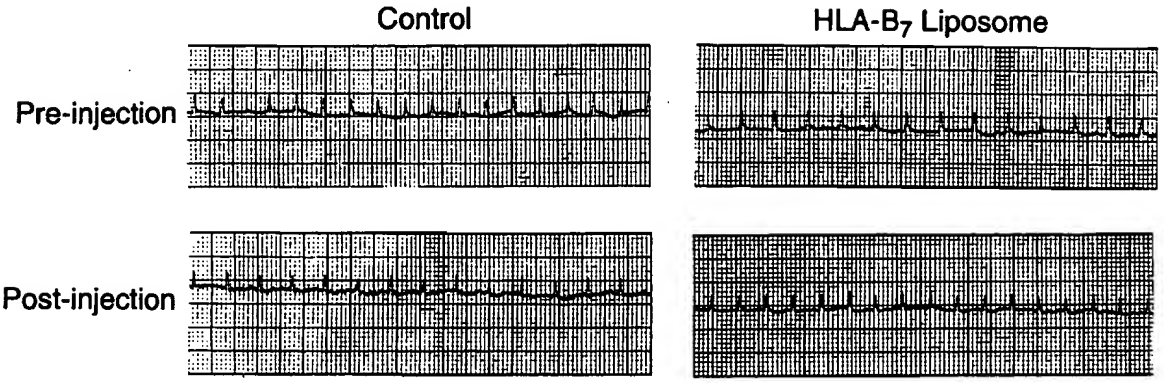

FIG. 3. Effect of DNA–liposome infusions on cardiac rate and rhythm measured by electrocardiography. Five mice were monitored electrocardiographically before and after intravenous infusions of saline (control, $n = 2$) or HLA-B7 liposomes ($n = 3$). Female BALB/c mice were anesthetized by metathane inhalation. Surface electrodes were attached to the four limbs, and surface lead I or II was recorded continuously at 50 mm/sec paper speed. Representative tracings are shown for each group pre-injection and 5 min post-injection.

In theory, a variety of such vectors could be employed for gene transfer *in vivo*, including DNA–liposome complexes, naked plasmid DNA, protein/DNA, or inactivated viral DNA complexes. In this study, we examine the toxicities of a novel DNA–liposome complex that may provide for a substantial increase in the amount of recombinant DNA which could be administered *in vivo*. In this formulation, the cationic lipid, DMRIE, has been substituted for DC-Chol. The resulting DNA–liposome complex has enhanced transfection efficiencies *in vitro* (Fig. 1). Although the magnitude of this effect is relatively small (two- to seven-fold), these plasmids do not aggregate at higher cell concentrations, and a substantially higher quantity of plasmid can be introduced *in vivo* by this method. This DNA–liposome also has the ability to facilitate an antitumor response in a case where the other liposome is less active (Fig. 2).

The ability to introduce genes at higher concentrations could enhance gene transfer and stimulate increased synthesis of gene products that are required for therapeutic effects. For example, recently, high-level concentrations of Lipofectin (BRL, Gaithersburg, MD) and plasmid expression vectors have been described that allow for gene expression systemically following intravenous infection (Zhu *et al.*, 1993). Additional Phase 1 studies will be required in humans to test this possibility. This study, together with previous studies that have demonstrated the relative safety of other DNA–liposome complexes (Nabel *et al.*, 1992a; Stewart *et al.*, 1992), provides evidence that the administration of such compounds *in vivo* is well tolerated.

The present study confirms that this treatment is tolerated at doses up to 1,000-fold higher than previously analyzed and has the potential to deliver larger quantities of genes. As in previous studies, although DNA was detected in the myocardium following intravenous injection, there was no effect on myocardial function, either acutely or chronically. Therefore, the intravenous infusion of DNA–liposome complexes continues to appear safe with regard to cardiac function even at higher doses. Finally, the concern was raised regarding the potential for inadvertent introduction of DNA into germ cells. We found that DNA was not detected in gonadal tissue, following gene transfer with DNA/DC-Chol complexes, even by PCR (Nabel *et al.*, 1992a). The previous study used lower doses of the DNA–liposome complex. The present study also confirms that the lack of recombinant DNA in gonadal tissue at concentrations up to 1,000-fold greater than those reported previously. Taken together, these data suggest that several cationic lipids may be useful for human gene therapy and are unlikely to cause significant toxicity at these doses, even when modifications are made in one of the charged lipid components. These data also suggest that additional modifications of the lipid can be incorporated in the future that will facilitate the efficiency of targeting DNA–liposome complexes to specific tissues *in vivo*.

TABLE 6. MEASUREMENTS OF TOTAL CPK (U/L) BEFORE AND AFTER INTRAVENOUS INJECTION WITH DNA–LIPOSOMES IN MICE

Plasmid (μg):	*0.5*	*5*	*50*
DMRIE/DOPE (nmol):	*1.5*	*15*	*150*
Pre-injection	339 ± 122	156 ± 44	156 ± 44
Post-injection	189 ± 63	163 ± 44	163 ± 44
	$p \leq 0.79$	$p \leq 0.96$	$p \leq 0.31$

Serum samples were obtained from BALB/c female mice prior to intravenous injection and 16 hr following injection of HLA-B7 liposome complexes ($n = 5$, each dose). Serum was analyzed by Roche Biomedical Laboratory (Burlington, NC).

REFERENCES

FELGNER, J.H., KUMAR, R., SRIDHAR, C.N., WHEELER, C.J., TSAI, Y.J., BORDER, R., RAMSEY, P., MARTIN, M., and FELGNER, P.L. (1993). Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations—part 1. J. Biol. Chem. (in press).

GAO, X., and HUANG, L. (1991). A novel cationic liposome reagent for efficient transfection of mammalian cells. Biochem. Biophys. Res. Commu. **179**, 280–285.

NABEL, E.G., PLAUTZ, G., BOYCE, F.M., STANLEY, J.C., and NABEL, G.J. (1989). Recombinant gene expression in vivo within endothelial cells of the arterial wall. Science **244,** 1342–1344.

NABEL, E.G., PLAUTZ, G., and NABEL, G.J. (1990). Site-specific gene expression in vivo by direct gene transfer into the arterial wall. Science **249,** 1285–1288.

NABEL, E.G., GORDON, D., YANG, Z-Y., XU, L., SAN, H., PLAUTZ, G.E., WU, B-Y., GAO, X., HUANG, L., and NABEL, G.J. (1992a). Gene transfer in vivo with DNA-liposome complexes: lack of autoimmunity and gonadal localization. Hum. Gene Ther. **3,** 649–656.

NABEL, E.G., PLAUTZ, G., and NABEL, G.J. (1992b). Transduction of a foreign histocompatibility gene into the arterial wall induces vasculitis. Proc. Natl. Acad. Sci. USA **89,** 5157–5161.

NABEL, E.G., YANG, Z., LIPTAY, S., SAN, H., GORDON, D., HAUDENSCHILD, C.C., and NABEL, G.J. (1993a). Recombinant platelet-derived growth factor B gene expression in porcine arteries induces intimal hyperplasia in vivo. J. Clin. Invest. **91,** 1822–1829.

NABEL, E.G., YANG, Z.Y., PLAUTZ, G., FOROUGH, R., ZHAN, X., HAUDENSCHILD, C.C., MACIAG, T., and NABEL, G.J. (1993b). Recombinant fibroblast growth factor-1 gene expression promotes intimal hyperplasia and angiogenesis in arteries in vivo. Nature **362,** 844–846.

NABEL, G.J., CHANG, A., NABEL, E.G., PLAUTZ, G., FOX, B.A., HUANG, L., and SHU, S. (1992a). Clinical Protocol: Immunotherapy of malignancy by in vivo gene transfer into tumors. Hum. Gene Ther. **3,** 399–410.

NABEL, G.J., CHANG, A., NABEL, E.G., PLAUTZ, G., FOX, B.A., HUANG, L., and SHU, S. (1992b). Clinical Protocol: Response to the points to consider for immunotherapy of malignancy by in vivo gene transfer into tumors. Hum. Gene Ther. **3,** 705–711.

NABEL, G.J., NABEL, E.G., YANG, Z.-Y., FOX, B.A., PLAUTZ, G.E., GAO, X., HUANG, L., SHU, S., GORDON, D., and CHANG, A.E. (1993). Direct gene transfer with DNA liposome complexes in melanoma: Expression, biologic activity and lack of toxicity in humans. Proc. Natl. Acad. Sci. USA (in press).

PLAUTZ, G.E., YANG, Z., WU, B., GAO, X., HUANG, L., and NABEL, G.J. (1993). Immunotherapy of malignancy by in vivo gene transfer into tumors. Proc. Natl. Acad. Sci. USA **90,** 4645–4649.

STEWART, M.J., PLAUTZ, G.E., DEL BUONO, L., YANG, Z.Y., XU, L., GAO, X., HUANG, L., NABEL, E.G., and NABEL, G.J. (1992). Gene transfer in vivo with DNA-liposome complexes: safety and acute toxicity in mice. Hum. Gene Ther. **3,** 267–275.

ZHU, N., LIGGITT, D., LIU, Y., and DEBS, R. (1993). Systemic gene expression after intravenous DNA delivery into adult mice. Science **261,** 209–211.

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Systemic Gene Expression After Intravenous DNA Delivery into Adult Mice

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Direct gene transfer into adult animals resulting in generalized or tissue-specific expression would facilitate rapid analysis of transgene effects and allow precise in vivo manipulation of biologic processes at the molecular level. A single intravenous injection of expression plasmid:cationic liposome complexes into adult mice efficiently transfected virtually all tissues. In addition to vascular endothelial cells, most of the extravascular parenchymal cells present in many tissues including the lung, spleen, lymph nodes, and bone marrow expressed the transgene without any apparent treatment-related toxicity. The transgene was still expressed in large numbers of cells in multiple tissues for at least 9 weeks after a single injection. Expression could be targeted to specific tissues and cell types, depending on the promoter element used.

The ability to efficiently transfect large numbers and diverse populations of somatic cells in adult animals would provide researchers a rapid and reproducible source of animals with which to analyze the function of genes transferred and expressed in vivo. This ability could also create new opportunities for directly altering gene expression in living hosts. (i) Loss or gain of function phenotypes could be produced directly in adult animals. (ii) The effects of expressing a transgene at different developmental stages could be assessed. (iii) Transgenes whose expression is lethal during embryogenesis could be expressed after birth. (iv) Multiple transgenes could be expressed in series in the same animal. Cationic liposomes containing *N*[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) have been used to deliver DNA (*1*), mRNA (*2*), or proteins (*3*) into cultured cells. However, their usefulness as an in vivo DNA carrier system has been limited because the expression of transferred genes has been confined to small numbers of cells within a single tissue (*4*, *5*).

By intravenously injecting various mixtures of a cytomegalovirus (CMV)–chloramphenicol acetyltransferase (CAT) expression plasmid and DOTMA:dioleoyl phosphatidylethanolamine (DOPE) liposomes, we determined that a ratio of 1 μg of plasmid DNA to 8 nmol of liposomal lipid produced maximal CAT gene expression in lung, heart, and lymph node tissues (Fig. 1A). The ratio of DNA to cationic liposomes determines the net surface charge on the complex, which can alter the interactions of the complex with potential opsonins in the circulation (*6*) and alter the ability of the complex to bind and enter cells in vivo. Using the DNA-to-liposome ratio (1:8), we found that doses of more than 50 μg of the CMV-CAT plasmid per 20-g mouse produced generalized CAT gene expression throughout the body (Fig. 1B). With a dose of 100 μg of DNA, high levels of CAT activity (*7*) were present in the lung, spleen, liver, heart, kidney, and lymph nodes (Fig. 1B) as well as in the thymus, uterus, ovary, skeletal muscle, pancreas, bone marrow, stomach, small intestine, and colon (*8*). When tissues were assayed for CAT activity per milligram of tissue protein, as performed for Fig. 1B, the lung and spleen had the highest levels of CAT activity (*9*).

In addition to the DNA-to-liposome ratio and DNA dose, conditions which increased levels of transgene expression in vivo were the use of the immediate–early 1 promoter–enhancer element of the human CMV and an intron 5′ to the coding region rather than a 3′ intron (*10*). Similarly, constructs containing the human CMV promoter (*11*) and an intron 5′ to the coding sequence (*12*) significantly increased the level and extent of gene expression in transgenic mice after oocyte injection.

We extracted genomic DNA from mouse tissues and subjected it to both Southern (DNA) blot and polymerase chain reaction (PCR) analyses to probe for the presence of the CAT gene. Significant amounts of plasmid DNA were present in DNA extracted from the lung and liver at day 1 but were undetectable by Southern analysis at day 21 (Fig. 2A). We detected intact plasmid DNA in supercoiled, relaxed, and linearized forms by Southern analysis at day 1 (Fig. 2A), demonstrating that nonintegrated plasmid was present. The CAT gene was detected by PCR analysis in the lung, lymph nodes, heart, and spleen of treated animals at 63 days after injection (Fig. 2B). Transient CAT gene expression in the lung after intravenous (iv) injection has been reported (*4*). Prolonged

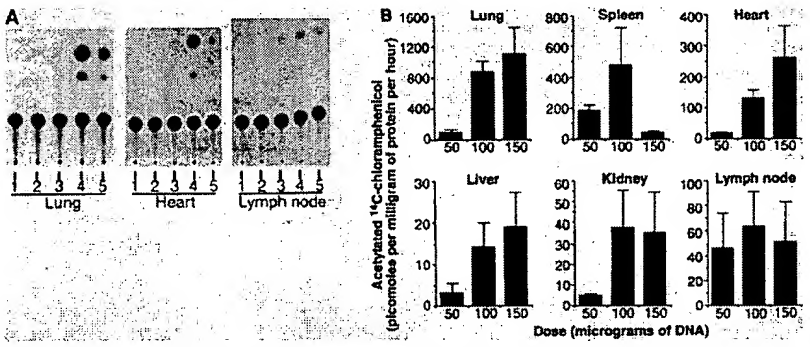

Fig. 1. The effect of the DNA-to-liposome ratio on CAT gene expression in vivo. (**A**) Female ICR mice (20 g) (Simonson) were injected in their tail veins with 100 μg of a human CMV-CAT plasmid (pCIS-CAT, obtained from C. Gorman, Genentech) complexed to 200 (lane 2), 600 (lane 3), 800 (lane 4), or 1200 nmol (lane 5) of DOTMA:DOPE liposomes in 200 μl of 5% dextrose in water. (Lane 1, untreated control.) We prepared liposomes from DOTMA and DOPE in a 1:1 molar ratio as described (*3*). Mice were killed 48 hours later, and tissues were harvested and homogenized. We prepared extracts and assayed for protein by means of a Coomasie blue–based assay (Bio-Rad). Each tissue was normalized for protein and assayed for CAT activity as described (*21*), with the following modification: 0.3 μCi of ^{14}C-labeled chloramphenicol (55 mCi/mmol) was added to 200 nmol of acetyl coenzyme A for a final volume of 122 μl. (**B**) The effects of the injected dose of DNA:liposome complexes on CAT gene expression in vivo. We gave ICR mice (in groups of three) a tail vein injection of 50, 100, or 150 μg of plasmid DNA complexed to 400, 800, or 1200 nmol of DOTMA:DOPE liposomes, respectively, and killed the mice 48 hours later. Paraoxon (1 mM) was added to liver and kidney extracts. To quantitate CAT activity in tissue extracts, we cut areas corresponding to [^{14}C]chloramphenicol and its acetylated derivatives from the thin-layer chromatography plate and counted them in a scintillation counter. The enzymatic activity of CAT in tissue extracts was expressed as picomoles of [^{14}C]chloramphenicol acetylated per milligram of protein per hour under assay conditions. Values represent mean ± SD.

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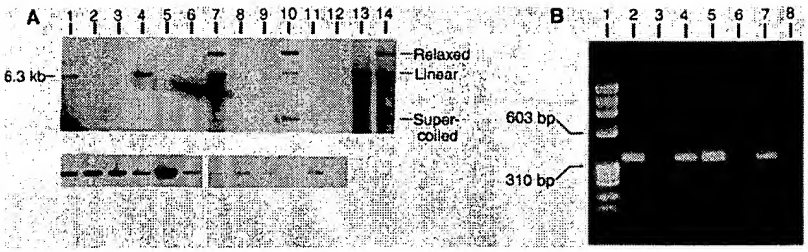

Fig. 2. Southern and PCR analyses to detect the CAT gene in mouse tissues. (**A**) We gave mice iv injections of DNA:liposome complexes or left the mice untreated, then 1 or 21 days later we harvested tissues and isolated nuclear DNA (*22*). For Southern analysis, DNA was digested with Eco RV, which does not cut within the plasmid, and Bgl II, which cuts at a single site within the plasmid. The DNA fragments were separated by electrophoresis on a 1% agarose gel and transferred to Hybond-N+ nylon membranes (Amersham). The blot was hybridized with a ^{32}P-labeled Bam H1–Pst 1 fragment of the mouse β-globin gene, washed, and rehybridized with the CMV-CAT plasmid. Lanes 1 through 6 were cut with Bgl II: (lane 1) day-1 lung; (lane 2) day-21 lung; (lane 3) control lung; (lane 4) day-1 liver; (lane 5) day-21 liver; and (lane 6) control liver. Lanes 7 through 12 were cut with Eco RV: (lane 7) day-1 lung; (lane 8) day-21 lung; (lane 9) control lung; (lane 10) day-1 liver; (lane 11) day-21 liver; and (lane 12) control liver. The remaining two lanes contain (lane 13) CAT plasmid–Bgl II and (lane 14) CAT plasmid–Eco RV. (**B**) Mouse DNA extracted from the lung, lymph nodes, heart, and spleen of an animal injected 63 days before, as well as DNA extracted from the spleen of a control mouse, was subjected to PCR analysis using two primers specific for the CAT gene, ACGTTTCAGTTTGCTCATGG and AGCTAAGGAAGCTAAAATGG, which yield 320-bp fragments. We performed the PCR analysis in a final volume of 100 μl of 2.5 mM $MgCl_2$ with 100 pmol of each primer. The cycles were 94°C for 30 s, 50°C for 1 min, and 72°C for 1 min. After 27 cycles we analyzed the products on 1.6% agarose gels. Lane 1, øX174 ladder (BRL); lane 2, day-63 lung; lane 3, day-63 lymph node; lane 4, day-63 heart; lane 5, day-63 spleen; lane 6, control spleen; lane 7, CAT plasmid; and lane 8, water control.

Table 1. Immunohistochemical localization of CAT antigen in tissues harvested 24 to 48 hours after iv injection with CMV-CAT:cationic liposome complexes. Initial evaluations were always conducted in a blinded fashion.

Organ	Tissues staining positive for CAT antigen	Relative expression of CAT antigen in tissues*
Lung	Alveolar walls†	4
	Bronchioles	3
Heart	Endothelium	3
	Myocytes	1
Bone marrow	Mononuclear cells	2
Liver	Endothelium	1
	Hepatocytes	1
Spleen	Red pulp	4
	White pulp	3
Lymph node	Paracortex	4
	Germinal center	1
Kidney	Endothelium	2
Lung‡	Metastatic tumor cells	2–3

*A semiquantitative estimation of the frequency of positively stained cells in a representative series of immunohistochemical preparations (range: 1, staining of <25% of cells; to 4, staining of >75% of cells). These results were compiled from a minimum of three different experiments consisting of a total of at least 10 animals, and all the results were consistent. †Includes endothelium and alveolar lining cells (indistinguishable). ‡From mice bearing B16 melanoma lung metastases.

expression of episomal transgenes in nondividing cell types has been reported after intramuscular injection of naked DNA into mice (*13*) or after cationic liposome–mediated transfection of freshly isolated cells from rodent lung (*14*).

We next assessed by immunohistochemistry the types and numbers of cells transfected in vivo after iv injection. Although circulating DNA:liposome complexes would be expected to be confined to the vascular compartment, we found that iv injection of CMV-CAT:liposome complexes transfected the majority of all cells present in the lung, including most of the alveolar and airway parenchymal cells, as well as cells of the vascular endothelial lining cells (Table 1). The staining of both alveolar and endothelial cells was diffuse (Fig. 3A). Staining intensity varied throughout the lung, but negatively staining areas were rare.

Because CAT activity was present in large numbers of extrapulmonary tissues after iv injection of CMV-CAT:liposome complexes, we assessed the cellular pattern of CAT gene expression by immunohistochemistry in several other tissues as well. Two patterns of transgene expression were apparent: (i) generalized expression throughout the tissue (as seen in the lung) or (ii) expression largely confined to the vascular endothelial compartment. Specifically, CAT gene expression was present in large numbers of extravascular parenchymal cells in the spleen, liver, lymph nodes, and bone marrow after injection into normal animals (Table 1 and Fig. 3C). [The lung and spleen had the largest amounts of CAT protein as revealed by immunostaining (Fig. 3 and Table 1) and by enzymatic activity measurements (Fig. 1B).] Thus, circulating DNA:liposome complexes appear to readily extravasate across vascular endothelial barriers in these tissues. In contrast with this diffuse pattern of parenchymal cell transfection, CAT gene expression was largely confined to the vascular compartment in the heart and kidney. In these tissues, most endothelial cells lining small blood vessels, but few extravascular parenchymal cells, were transfected (Table 1).

We also tested the ability of the CMV-CAT:cationic liposome complexes to be taken up by and expressed in parenchymal lung tumors. These complexes were intravenously injected into mice bearing established B16 melanoma lung metastases. We found that both metastatic lung tumors and tumor emboli within the pulmonary vascular compartment were efficiently transfected (Table 1).

Next we assessed the duration of CAT gene expression in tissues by immunohistochemistry. Expression of the CAT gene, as detected by positive staining, was still present in approximately 40% of all lung cells, as well as in approximately 30% of lymph node and spleen cells, 9 weeks after a single iv injection of CMV-CAT:liposome complexes (*8*). The half-life of CAT activity in freshly isolated rodent hepatocytes transfected with the CAT gene ex vivo is approximately 20 hours (*15*). Thus, the persistence of CAT activity does not appear to result from stability of the CAT protein within cells. Furthermore, iv injections of DNA:liposome complexes repeated at 2-week intervals produced peak levels of transgene expression comparable to those produced by the first dose (*8*).

We have also tested the expression of the human gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) driven by the human CMV promoter. Using immunohistochemistry, we found that the human CFTR transgene is still expressed in large numbers of lung cells for at least 150 days after the last iv injection of CMV-CFTR plasmid:cationic liposome complexes into mice (*16*). These results indicate that transgene expression can persist in tissues for prolonged periods of time after cationic liposome–based in vivo gene delivery.

Sections from a wide variety of tissues from treated animals were also analyzed for potential toxicity. We observed no treatment-related toxicity in any treated animal. Specifically, hematoxylin- and eosin-stained histologic sections from treated animals were indistinguishable from comparable sections from untreated animals, and organ weights were not significantly different between control and treated animals. Furthermore, treated mice appeared normal

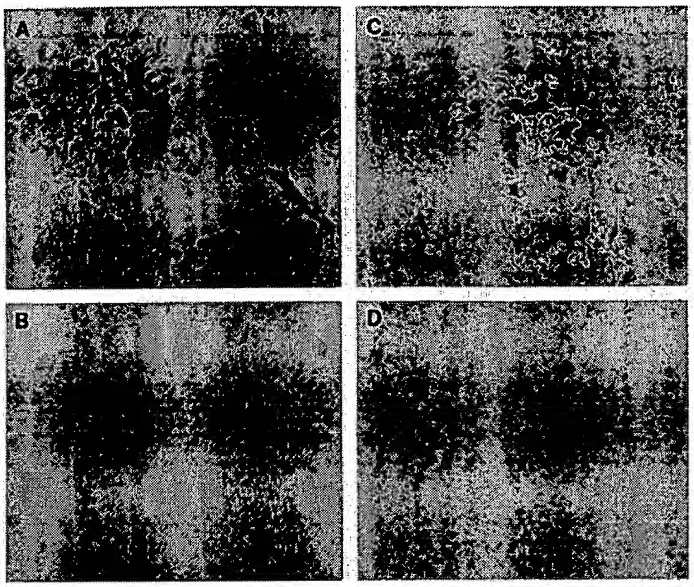

Fig. 3. Immunohistochemical analysis to detect CAT enzyme in mouse tissues. We injected ICR mice intravenously with 100 μg of CMV-CAT complexed to cationic liposomes, as described in Fig. 1B, or left the mice untreated and then killed them 48 hours later. For tumor localization studies, female C57B mice were injected intravenously with 5×10^4 B16 melanoma cells, received 14 days later 100 μg of the DNA:liposome complex or no treatment, and then killed 24 hours later. We then prepared the appropriate tissues for analysis (*23*). Red staining indicates the location of CAT antigen. (**A**) and (**B**) are adjacent lung sections from a mouse treated with CMV-CAT:liposome complexes. (**C**) and (**D**) are spleen sections from a CMV-CAT:liposome–treated mouse and a control mouse, respectively. To verify the specificity of CAT immunostaining, we first adsorbed the primary rabbit polyclonal anti-CAT against purified CAT (Sigma). For the liquid phase absorption we diluted the rabbit primary antibody 1:300 with a solution of purified CAT (CAT diluted 1:50 with a solution of PBS containing 0.5% casein and 2% normal goat serum) (B). Nonabsorbed antibody was similarly prepared but without the addition of purified CAT (A). The solutions were incubated for 1 hour at 37°C, placed on ice for an additional hour, and then subjected to immunostaining. The positive CAT immunostaining signal (A) is specifically blocked by absorbing the anti-CAT with purified CAT enzyme (B).

from the time we injected them with DNA:liposome complexes until they were killed. In the absence of inflammation or injury, the ability of circulating DNA:liposome complexes to extravasate into many of these tissues cannot be explained by altered vascular permeability. Consistent with our findings, other investigators have concluded that the systemic injection of DNA:cationic liposome complexes into animals appears nontoxic (*17*).

This somatic cell transgenic model provides simple, rapid, and reproducible transfer and expression of heterologous genes directly in adult animals. It could facilitate the identification of cis-acting sequences that regulate gene expression in vivo. Because the expression plasmid appears to be present primarily in episomal form, the use of these somatic cell transgenic animals may avoid unpredictable regulatory effects resulting from integration of transgenes into random sites within chromosomal DNA after the plasmid is injected into oocytes (*18*). Furthermore, specific tissues and cell types can be targeted in vivo by several, potentially complementary approaches. These include (i) using promoter-enhancer elements that are tissue- and cell type–specific, (ii) administering the plasmid regionally into selected tissue compartments (*19*), and (iii) coupling targeting ligands to the liposome surface (*20*). High level, generalized or tissue-specific expression of heterologous genes directly in adult animals could facilitate both the study and the control of molecular events in vivo and the range of diseases that can be treated by gene therapy.

REFERENCES AND NOTES

1. P. Felgner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7413 (1987).
2. R. Malone, P. Felgner, I. Verma *ibid.* **86**, 6077 (1989).
3. R. Debs *et al.*, *J. Biol. Chem.* **265**, 10189 (1990).
4. K. L. Brigham *et al.*, *Am. J. Med. Sci.* **298**, 278 (1989).
5. E. Nabel, G. Plautz, G. Nabel, *Science* **249**, 1285 (1990).
6. R. L. Juliano, in *Liposomes*, M. J. Ostro, Ed. (Dekker, New York, 1983), pp. 53–86.
7. CAT activity produced per gram of tissue extract was approximately 1 to 2% of CAT activity produced per gram of cell extract. The cell extracts were prepared from several highly transfectable cell lines that we transfected in culture with CMV-CAT:DOTMA:DOPE liposome complexes. (Significant amounts of extracellular, and therefore nontransfectable, protein are present in tissue extracts, but not in cultured cells.) Furthermore, the amount of CAT activity in lung tissue from intravenously treated animals was within a factor of 3 of that produced by cells transfected in culture [single-cell suspensions prepared from whole lungs of untreated mice (*8*)]. This suggests that the process of in vivo transfection is relatively efficient.
8. Data not shown.
9. We injected 100 μg of plasmid DNA complexed to 800 nmol of cationic liposomes per mouse for subsequent experiments. The 100-μg dose of CMV-CAT DNA contained $\sim 1.8 \times 10^{13}$ DNA molecules. These were associated with $\sim 4 \times 10^{12}$ cationic liposomes, with a mean liposome diameter of ~100 nm (*3*). Estimating that a 20-g mouse contains $\sim 2 \times 10^{10}$ cells [I. F. Tannock, in *Cancer: Principles and Practice of Oncology*, V. DeVita, S. Hellman, S. Rosenberg, Eds. (Lippincott, Philadelphia, PA, ed. 3, 1989), p. 4], we injected each mouse with $\sim 1 \times 10^9$ CMV-CAT DNA molecules per mouse cell.
10. N. Zhu and R. Debs, unpublished data.
11. E. V. Schmidt, G. Christoph, R. Zeller, P. Leder, *Mol. Cell. Biol.* **10**, 4406 (1990).
12. R. D. Palmiter, E. P. Sandgren, M. R. Avarbock, D. D. Allen, R. L. Brinster, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 478 (1991); T. Choi *et al.*, *Mol. Cell. Biol.* **11**, 3070 (1991).
13. J. A. Wolff *et al.*, *Science* **247**, 1465 (1990); G. Acsadi *et al.*, *Nature* **352**, 815 (1991).
14. R. Debs *et al.*, *Am. J. Respir. Cell Mol. Biol.* **7**, 406 (1992).
15. W. Jarnagin, R. Debs, M. Bissell, *Nucleic Acids Res.* **20**, 4205 (1992).
16. N. Zhu, D. Liggitt, Y. Liu, J. Cohn, R. Debs, unpublished data.
17. R. Stewart *et al.*, *Human Gene Ther.* **3**, 267 (1992); E. Nabel *et al.*, *ibid.*, p. 649.
18. R. Jaenisch, *Science* **240**, 1468 (1988); G. T. Merlino, *FASEB J.* **5**, 2996 (1991).
19. C. E. Holt, N. Garlick, E. Cornel, *Neuron* **4**, 203 (1990); R. Stribling *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11277 (1992).
20. R. Debs. T. Heath, D. Papahadjopoulos, *Biochim. Biophys. Acta* **901**, 183 (1987); K. Maruyama, S. J. Kennel, L. Huang, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5744 (1990).
21. C. M. Gorman, G. T. Merlino, M. C. Willingham, I. Pastan, B. H. Howard, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6777 (1982).
22. S. C. Feinstein, S. R. Ross, K. R. Yamamoto, *J. Mol. Biol.* **156**, 549 (1982).
23. Tissues were quick frozen, embedded in O.C.T. (Miles), sectioned at 6 μm, collected onto sialinized glass slides, fixed for 10 min in 4°C acetone, and washed in a solution of 0.1% Tween-20 in phosphate-buffered saline (PBS). Tissue sections were blocked with 10% goat serum, blotted, and incubated for 24 hours with a polyclonal antibody to CAT (anti-CAT) or a nonspecific control antibody at the appropriate dilution. The slides were washed, incubated for 60 min with a biotinylated antibody directed against the primary antibody, washed, then incubated with a streptavidin–alkaline phosphatase or peroxidase complex for 60 min, and washed again. Lastly, we applied a substrate-chromagen appropriate for the enzyme label (Zymed). Endogenous alkaline phosphatase activity was inhibited with levamisole. Spurious binding of streptavidin was blocked by application of free avidin and biotin (Zymed).

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